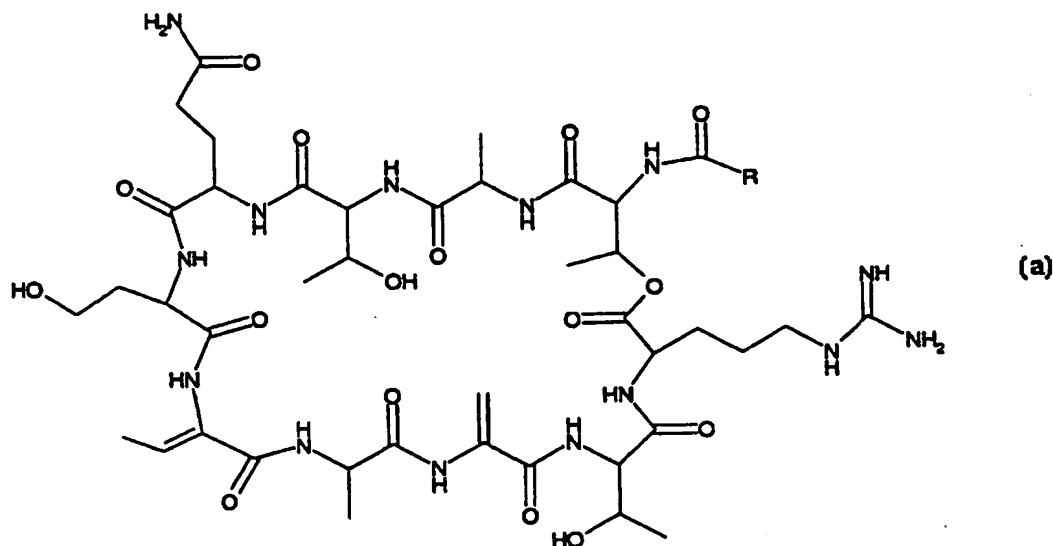




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(54) Title: ANTIFUNGAL AGENTS ISOLATED FROM *PSEUDOMONAS SYRINGAE*

(57) Abstract

The invention relates to *P. syringae* depsidecapeptides, method for making such peptide, and methods employing antifungal activity of these peptides. The *P. syringae* depsidecapeptides include a compound having formula (a) where R is a lipophilic moiety, or a pharmaceutically acceptable salt, ester, or hydrate thereof.

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ANTIFUNGAL AGENTS ISOLATED FROM *PSEUDOMONAS SYRINGAE*

5

FIELD OF THE INVENTION

The present invention relates to *P. syringae* depsidecapeptides, method for making such peptide, and methods employing antifungal activity of these peptides.

10

BACKGROUND

Fungal infections are a significant cause of disease, degradation of quality of life, and mortality among humans, particularly for immune compromised patients. The incidence in fungal infections in humans has increased greatly in the past 20 years. This is in part due to increased numbers of people with immune systems weakened or devastated by organ transplants, cancer chemotherapy, AIDS, age, and other similar disorders or conditions. Such patients are prone to attack by fungal pathogens that are prevalent throughout the population but are kept in check by a functioning immune system. These pathogens are difficult to control because some existing antifungal agents are either highly toxic or only inhibit fungal activity. For example, the polyenes are fungicidal but toxic; whereas, the azoles are much less toxic but only fungistatic. More importantly, there have been recent reports of azole and polyene resistant strains of *Candida* which severely limits therapy options against such strains.

Pseudomonas syringae produce several classes of antifungal or antibiotic agents, such as the pseudomycins, syringomycins, syringotoxins, and syringostatins, which are lipodepsinona peptides. Natural strains and transposon generated mutants of *P. syringae* produce these lipodepsinona peptides. Several of the pseudomycins, syringomycins and other lipodepsi peptide antifungal agents have been isolated, chemically characterized, and shown to possess wide spectrum antifungal activity, including activity against important fungal pathogens in both humans and plants. The pseudomycins, the syringomycins, the

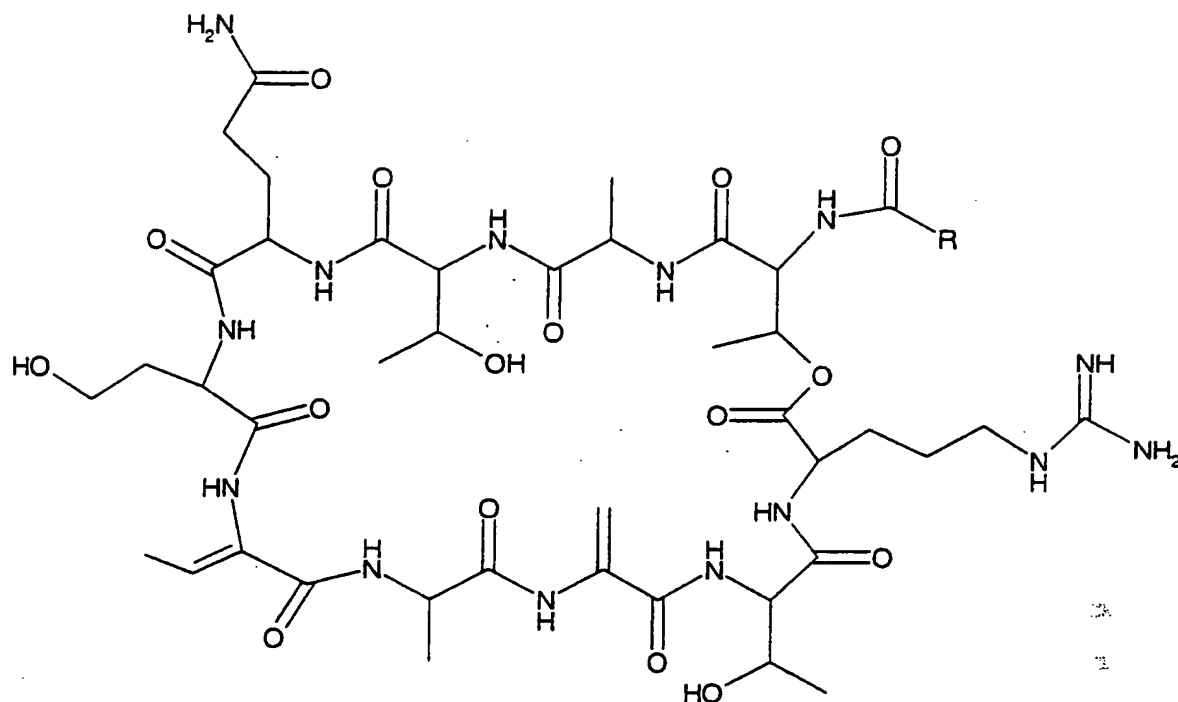
syringotoxins, and the syringostatins represent structurally distinct families of antifungal compounds.

None of the *P. syringae* lipodepsinonapeptides has been brought to market for antifungal therapy. Discovery of undesirable side effects, making formulations, scaling
5 up production, and other development problems have thus far prevented exploitation of the *P. syringae* lipodepsinonapeptides against the full range of fungal infections that affect animals, humans and plants. There remains a need for an antifungal agent that can be used against infections not treated by existing antifungal agents and for application against infections in animals, humans, or plants.

10

SUMMARY OF THE INVENTION

The present invention provides a depsidecapeptide produced by *P. syringae* which contains the unusual amino acids homoserine (Hse), dehydroaminobutyric acid (Dhb) and dehydroalanine (Dha) as part of a depsidecapeptide ring. More specifically, the *P.*
15 *syringae* depsidecapeptide includes a depsidecapeptide ring having the amino acids, arginine, threonine, homoserine, dehydroaminobutyric acid, and dehydroalanine, and a lactone formed from a carboxyl group of the arginine and a hydroxyl group of the threonine. As isolated from *P. syringae*, the depsidecapeptide is a lipodepsidecapeptide: a cyclic peptide coupled to a lipophilic moiety. Typically the lipophilic moiety is a fatty
20 acid moiety coupled to the amino group of the threonine by an amide bond. Preferably, the fatty acid moiety is an *n*-dodecanoic acid moiety. The lipodepsidecapeptide is represented by formula I:



I

where R is a lipophilic moiety. The lipophilic moiety includes C₉-C₁₅ alkyl, C₉-C₁₅
 5 hydroxyalkyl, C₉-C₁₅ dihydroxyalkyl, C₉-C₁₅ alkenyl, C₉-C₁₅ hydroxyalkenyl, or C₉-C₁₅
 dihydroxyalkenyl. Preferably, the lipophilic moiety is C₁₁ alkyl. The alkyl, hydroxyalkyl,
 dihydroxyalkyl, alkenyl, hydroxyalkenyl, or dihydroxyalkenyl groups may be branched or
 unbranched. Preferably, the amino acid sequence of the depsidecapeptide ring is
 threonine-alanine-threonine-glutamine-homoserine-dehydroaminobutyric acid-alanine-
 10 dehydroalanine-threonine-arginine, referred to herein as “25-B1 decapeptide” or
 “Thr-Ala-Thr-Gln-Xaa-Xaa-Ala-Xaa-Thr-Arg (SEQ ID NO: 1)”.

As used herein, the term “25-B1 decapeptide antifungal agent A” refers to the specific
 depsidecapeptide having the preferred amino acid sequence SEQ ID NO: 1 and R =
 unbranched C₁₁ alkyl (i.e., R = -(CH₂)₁₀CH₃).

15 The invention also relates to methods employing a *P. syringae* depsidecapeptide
 for inhibiting fungal activity or for reducing the symptoms of a fungal infection in a
 patient in need thereof. Such methods can kill the fungus, decrease the burden of a fungal

infection, reduce fever and increase general well being of a patient. Consequently, the *P. syringae* depsidecapeptides may be used in the manufacture of a medicament for treatment of a patient as described herein. The methods and medicaments of the invention are effective against fungi such as *Candida parapsilosis*, *Candida albicans*,
5 *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

The invention provides using microorganisms in a method for producing an anti-fungal agent, such as the *P. syringae* depsidecapeptides described above and including a 25-B1 decapeptide. The method involves culturing *Pseudomonas syringae* in media including three or fewer amino acids and recovering one or more *P. syringae*
10 depsidecapeptides from the culture. In one embodiment, *P. syringae* culture is in medium including glycine and a lipid, a potato product, or a combination thereof at a pH of about 4 to 6.5 until one or more *P. syringae* depsidecapeptides is produced at a concentration of at least about 10 $\mu\text{g/mL}$. In addition, the invention provides *P. syringae* depsidecapeptides prepared by the method described above.

15 The invention also provides a method for treating or preventing fungal growth in a plant whereby a fungus is contacted with a one or more of the *P. syringae* depsidecapeptides described above.

DETAILED DESCRIPTION

20 Lipodepsidecapeptide Antifungal Agents

As used herein "lipodepsidecapeptide antifungal agent" refers to an antifungal agent having a cyclic decapeptide ring closed by a lactone group and having an appended hydrophobic group, such as a fatty acid moiety. Lipodepsidecapeptide antifungal agents are produced by *Pseudomonas syringae*. A representative of this class of compounds, 25-
25 B1 decapeptide antifungal agent A, has been purified and its structure determined. As used herein the term "*P. syringae* lipodepsidecapeptide" refers to a lipodepsidecapeptide antifungal agent produced by *P. syringae*, and includes 25-B1 decapeptide antifungal agent A and related analogs.

P. syringae lipodepsidecapeptides share several structural features. For example,
30 each of these antifungal agents includes the unusual amino acids homoserine (Hse),

dehydroaminobutyric acid (Dhb) and dehydroalanine (Dha) as part of a depsidecapeptide ring. In each of the *P. syringae* lipodepsidecapeptides, a carboxyl group of an arginine residue linked to the hydroxyl group of the N-terminal threonine forms a lactone that closes the depsidecapeptide ring. The sequence of the depsidecapeptide ring of the *P.*

5 *syringae* lipodepsidecapeptide can be represented as:

Thr-Xaa-Xbb-Xcc-Hse-Dhb-Xdd-Dha-Xee-Arg

in which each of Xaa, Xbb, Xcc, Xdd, and Xee are individually naturally occurring amino acids. Unlike the pseudomycin natural products, the lipodepsidecapeptides of the present invention do not contain chlorothreonine which is suspected to be the cause for irritation
10 at the injection site of pharmaceutical formulations containing pseudomycin compounds.

The depsidecapeptide ring is linked to a lipophilic moiety, such as a fatty acid, through an amide bond with an amino group of the N-terminal threonine. The fatty acid generally includes 10, 12, 14, or 16 carbons, typically bearing zero, one or two hydroxyl groups. The fatty acid may be branched or unbranched and may also contain at least one
15 unsaturation. Preferred fatty acid moieties include an *n*-decanoic acid moiety, an *n*-decanoic acid moiety substituted with one or two hydroxyl groups, an *n*-dodecanoic acid moiety, an *n*-dodecanoic acid moiety substituted with one or two hydroxyl groups, an *n*-tetradecanoic acid moiety, or an *n*-tetradecanoic acid moiety substituted with one or two hydroxyl groups.

20

25-B1 Decapeptide Antifungal Agents

As used herein, "25-B1 decapeptide antifungal agent" refers to one or more members of a family of antifungal agents that has been isolated from the bacterium *Pseudomonas syringae*. A 25-B1 decapeptide antifungal agent is a *P. syringae*
25 lipodepsidecapeptide. Specifically, a 25-B1 decapeptide antifungal agent is a *P. syringae* lipodepsidecapeptide having a depsidecapeptide ring with the sequence:

Thr-Ala-Thr-Gln-Hse-Dhb-Ala-Dha-Thr-Arg (SEQ ID NO: 1)

Each of the 25-B1 decapeptide antifungal agents has the same cyclic peptide nucleus, but they differ in the hydrophobic side chain attached to this nucleus. The 25-B1 decapeptide
30 antifungal agents include 25-B1 decapeptide antifungal agent A.

The 25-B1 decapeptide antifungal agents include a fatty acid linked through an amide bond with the amino group of the N-terminal threonine. The fatty acid moiety of 25-B1 decapeptide antifungal agent A is an *n*-dodecanoic acid moiety.

5 Biological Activities of *P. syringae* Lipodepsidecapeptides

A *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, has several biological activities including killing and inhibiting activity of various fungi, such as fungal pathogens of plants and animals. In particular, a 25-B1 decapeptide antifungal agent is an active antimycotic agent against fungi that cause opportunistic
10 infections in immune compromised individuals. These fungi include *Cryptococcus neoformans*, *Histoplasma capsulatum* and various species of *Candida* including *C. parapsilosis* and *C. albicans*.

Pseudomonas syringae

15 *Pseudomonas syringae* include a wide range of bacteria that are generally associated with plants. Some of the *P. syringae* are plant pathogens, while others are only weakly pathogenic or are saprophytes. Many different isolates of *P. syringae* produce one or more cytotoxic agents that can help this bacterium survive in the wild where it must compete with fungi and other bacteria. The cytotoxic agents produced by *P. syringae*
20 include anti-fungal agents such as the *P. syringae* lipodepsidecapeptides, including 25-B1 decapeptide antifungal agent A, the pseudomycins, the syringomycins, the syringotoxins, and the syringostatins.

Isolated strains of *P. syringae* that produce one or more pseudomycins, syringomycins, syringotoxins, syringostatins are well-known to those skilled in the art.
25 Wild type strain MSU 174 and a mutant of this strain generated by transposon mutagenesis, MSU 16H (ATCC 67028) have been described in U.S. Patent No. 5,576,298, issued November 19, 1996 to G. Strobel et al.; Harrison et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," *J. Gen. Microbiology* **137**, 2857-2865 (1991); and
30 Lamb et al., "Transposon mutagenesis and tagging of fluorescent pseudomonas:

Antimycotic production is necessary for control of Dutch elm disease." Proc. Natl. Acad. Sci. USA **84**, 6447-6451 (1987). Methods for growth of various strains of *P. syringae* and their use in production of antifungal agents such as pseudomycins are also disclosed in U.S. Patent Application Serial No. _____ by Matthew D.

- 5 Hilton, et al. entitled "Pseudomycin Production By *Pseudomonas Syringae*" submitted eventdate herewith and described below. The disclosures of the references cited in this paragraph are incorporated herein by reference.

Strains of *P. syringae* that are suitable for production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be isolated
10 from environmental sources including plants such as barley plants, citrus plants, and lilac plants, and from forest floor litter, soil, water, air, and dust. The present invention includes a strain, an isolate, and a biologically-purified culture of *P. syringae* that produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, in amounts greater than about 10 $\mu\text{g/mL}$, preferably from at least about 10
15 $\mu\text{g/mL}$ to about 50 $\mu\text{g/mL}$. Preferably, the biologically-purified culture of a microorganism is of *Pseudomonas syringae* strains MSU 16H, 25-B1, 67H1, 7H9-1, or a pseudomycin-producing mutant, variant, isolate, or recombinant of these strains. Cultures of MSU 16H are on deposit at Montana State University (Bozeman, Montana, USA) and available from the American Type Culture Collection (Parklawn Drive,
20 Rockville, MD, USA) Accession No. ATCC 67028.

A strain of *P. syringae* that is suitable for production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be isolated from environmental sources including plants, such as barley plants, citrus plants, and lilac plants, and also from sources such as soil, water, air, and dust. A preferred strain is
25 isolated from plants. These environmental isolates of *P. syringae* can be referred to as wild type. As used herein, "wild type" refers to a dominant genotype which naturally occurs in the normal population of *P. syringae* (i.e., strains or isolates of *P. syringae* that are found in nature and not produced by laboratory manipulation). As is the case with other organisms, the characteristics of the lipodepsidecapeptide-producing cultures
30 employed in this invention. *P. syringae* strains such as MSU 174, MSU 16H, MSU 206,

25-B1, and 7H9 are subject to variation. Thus, progeny of these strains, e.g., recombinants, mutants and variants, may be obtained by methods well-known to those skilled in the art.

Mutant strains of *P. syringae* are also suitable for production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A. As used herein, "mutant" refers to a sudden heritable change in the phenotype of a strain, which can be spontaneous or induced by known mutagenic agents, including radiation and various chemicals. Mutant *P. syringae* of the present invention can be produced using a variety of mutagenic agents including radiation such as ultraviolet light, and x-rays; chemical mutagens; site-specific mutagenesis; and transposon mediated mutagenesis. Examples of chemical mutagens are ethyl methyl sulfonate (EMS), diepoxyoctane, N-methyl-N-nitro-N'-nitrosoguanine (NTG), and nitrous acid.

P. syringae suitable for producing one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, according to the present invention can be generated by treating the bacteria with an amount of a mutagenic agent effective to produce mutants that overproduce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, or that produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, under advantageous growth conditions. While the type and amount of mutagenic agent to be used can vary, a preferred method is to serially dilute NTG to levels ranging from about 1 to about 100 $\mu\text{g/mL}$. Preferred mutants of the invention are those that overproduce 25-B1 decapeptide antifungal agent A, and grow in minimal medium. The mutants overproduce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, preferably from at least about 10 $\mu\text{g/mL}$ to about 50 $\mu\text{g/mL}$.

Environmental isolates, mutant strains, and other desirable strains of *P. syringae* can be subjected to selection for desirable traits of growth habit, growth medium, nutrient source, carbon source, growth conditions, and amino acid requirements. Preferably, a strain of *P. syringae* producing *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, is selected for growth on minimal defined medium. Preferred strains exhibit the characteristics of producing one or more *P. syringae*

lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, when grown on a medium including glycine plus, optionally, a lipid, a potato product, or both.

Recombinant strains can be developed by transforming the *P. syringae* strains, using procedures well-known to those skilled in the art. Through the use of recombinant technology, the *P. syringae* strains can be transformed to express a variety of gene products in addition to the antibiotics these strains produce. For instance, one can transform the strains with a recombinant vector that confers resistance to an antibiotic to which the strains are normally sensitive. Transformants thus obtained will produce not only a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, but also the resistance-conferring enzyme that allows selection of the transformed from wild type cells. Furthermore, using similar techniques, one can modify the present strains to introduce multiple copies of the endogenous lipodepsidecapeptide-biosynthesis genes to achieve greater lipodepsidecapeptide yield. Progeny, i.e. natural and induced variants, mutants and recombinants, of the *P. syringae* strains 25-B1, 67H1, and 7H9-1 which retain the characteristic of lipodepsidecapeptide production are part of this invention.

Growth of *Pseudomonas syringae*

As described herein, "aqueous nutrient media" refers to a water-base composition including minerals and organic compounds and their salts necessary for growth of the bacterium used in the present invention. Preferred nutrient media contain an effective amount of three or fewer amino acids, preferably, glutamic acid, glycine, histidine, or a combination thereof. In one embodiment, the medium contains an effective amount of glycine and, optionally, one or more of a potato product and a lipid. Glycine can be provided as a single amino acid or as part of a mixture of amino acids, such as hydrolyzed protein. Suitable lipids include soybean oil, fatty acids, or fatty acid esters. Suitable potato products include potato dextrose broth, potato dextrin, potato protein, or a commercial mashed potato mix food product. Preferred minerals in the nutrient medium include salt mixtures typically used in cell culture and fermentation, such as Czapek mineral salts, which includes KCl, MgSO₄, and FeSO₄. Organic compounds in the nutrient media preferably includes glucose and can optionally include soluble starch; other

like organic compounds can also be included. The pH of the medium is preferably between about 4 and 6.5, more preferably about 4.5 to about 5.7, most preferably about 5.2.

Although the amount of each ingredient in the nutrient broth is not typically critical to growth of the bacteria or to production of a *P. syringae* lipodepsidecapeptide. such as 25-B1 decapeptide antifungal agent A, certain levels of nutrients are advantageous. A preferred amount of glycine is about 0.1 g/L to about 10 g/L, more preferably about 0.3 g/L to about 3 g/L, most preferably about 1 g/L. A preferred amount of lipid is about 1 g/L to about 10 g/L of an oil product such as soybean oil, more preferably about 0.5 g/L to about 2 g/L of soybean oil. A preferred amount of a fatty acid or fatty acid ester is about 0.5 g/L to about 5 g/L. Preferred amounts of potato products include about 12 g/L to about 36 g/L, more preferably about 24 g/L of potato dextrose broth; about 5 g/L to about 50 g/L, preferably about 30 g/L of a commercial mashed potato mix; about 1 g/L to about 30 g/L, preferably about 20 g/L of potato dextrin; and/or about 1 g/L to about 10 g/L, preferably about 4 g/L of potato protein. A preferred nutrient medium includes minerals, preferably, KCl at about 0.02 to about 2 g/L, more preferably about 0.2 g/L; MgSO₄, preferably MgSO₄•7H₂O, at about 0.02 to about 2 g/L, more preferably about 0.2 g/L; and FeSO₄, preferably FeSO₄•7H₂O, at about 0.4 to about 40 mg/L, more preferably about 4 mg/L. When present, soluble starch is preferably at about 0.5 to about 50 g/L, more preferably about 5 g/L. Glucose is preferably present at about 2 to about 80 g/L, more preferably about 20 g/L.

P. syringae are typically grown in the media described under conditions of controlled or regulated pH, and temperature. *P. syringae* grow and produce one or more cytotoxic agents at temperatures between about 15 °C and about 35 °C, preferably about 20 °C to about 30 °C, more preferably about 25 °C. *P. syringae* grow and produce one or more cytotoxic agents at pH between about 4 and about 9, more preferably between about 4 and about 6, most preferably from about 4.5 to about 5.5. Typically growth of *P. syringae* does not occur when the temperature is above about 37° C or below 10° C or when the pH is above about 9 or below about 4.

Method for Producing a *P. syringae* Lipodepsidecapeptide

To produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, from a wild type or mutant strain of *P. syringae*, the organism is cultured with agitation in an aqueous nutrient medium including an effective amount of three or fewer amino acids. The three or fewer amino acids are preferably glutamic acid, glycine, histidine, or a combination thereof. In one preferred embodiment, the amino acids include glycine and, optionally, one or more of a potato product and a lipid. Culturing is conducted under conditions effective for growth of *P. syringae* and production of a desired *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Effective conditions include a temperature of about 22° C to about 27° C, and a duration of about 36 hours to about 96 hours. When cultivated on media such as those described herein, *P. syringae* can grow at cell densities up to about 10-15 g/L dry weight and produce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, in a total amount at least about 10 µg/mL, preferably at least about 50 µg/mL.

Controlling the concentration of oxygen in the medium during culturing of *P. syringae* is advantageous for production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Preferably, oxygen levels are maintained at about 5% to about 50% saturation, more preferably about 30% saturation. Sparging with air, with pure oxygen, or with gas mixtures including oxygen can regulate the concentration of oxygen in the medium. Further, adjustment of the agitation rate can be used to adjust the oxygen transfer rate.

Controlling the pH of the medium during culturing of *P. syringae* is advantageous for production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. The pH of the culture medium can be maintained at less than about 6 and above about 4.

P. syringae can produce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, when grown in batch culture. However, fed-batch or semi-continuous feed of glucose and, optionally, an acid or base, such as ammonium

hydroxide, to control pH, enhances production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, by *P. syringae* can be further enhanced by using continuous culture methods in which glucose and, optionally, an acid or base, such as ammonium hydroxide, to control pH, are fed automatically. The pH is preferably maintained at a pH of about 5 to about 5.4, more preferably about 5.0 to about 5.2.

Choice of *P. syringae* strain can affect the amount and distribution of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, produced by culturing under the conditions described herein. For example, strain 25 B1 can produce predominantly 25-B1 decapeptide antifungal agent A.

The cyclic decapeptide nucleus of the *P. syringae* lipodepsidecapeptides can be prepared by cleaving off the lipophilic moiety, such as by deacylation. Cleavage and deacylation methods are well-known to those skilled in the art, such as the use of deacylase enzymes.

Formulation and Antifungal Action of *P. syringae* Lipodepsidecapeptides

A *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, shows *in vitro* and *in vivo* activity and is useful in combating either systemic fungal infections or fungal skin infections. Accordingly, the present invention provides a method of inhibiting fungal activity including contacting a *P. syringae* lipodepsidecapeptide, such as a 25-B1 decapeptide antifungal agent, or a pharmaceutically acceptable salt thereof, with a fungus. A preferred method includes inhibiting growth or activity of various fungi such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, and species of *Candida* including *C. parapsilosis* and *C. albicans*. As used herein "contacting" a compound of the invention with a parasite or fungus refers to a union or junction, or apparent touching or mutual tangency of a compound of the invention with a parasite or fungus. However, the term contacting does not imply any mechanism of inhibition.

The present invention further provides a method of treating a fungal infection which includes administering an effective amount of a *P. syringae* lipodepsidecapeptide,

such as a 25-B1 decapeptide antifungal agent, or a pharmaceutically acceptable salt thereof, to a host in need of such treatment. A preferred method includes treating an infection by various fungi such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, and strains of *Candida* including *C. parapsilosis* and *C. albicans*. When administered in
5 an effective antifungal amount, a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, reduces the burden of a fungal infection, reduces symptoms associated with the fungal infection, and can result in elimination of the fungal infection.

Some patients in need of antifungal therapy with a formulation of one or more *P.*
10 *syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, have severe symptoms of infection, such as high fever, and are likely to be in intensive or critical care. Various fungi can cause such serious infections. *Candida spp.*, for example, causes mucosal and serious systemic infections and may exist as azole- or polyene-resistant strains. *Aspergillus* causes life-threatening systemic infections. *Cryptococcus* is
15 responsible for meningitis. Such serious fungal infections may occur in immune compromised patients, such as those receiving organ or bone marrow transplants, undergoing chemotherapy for cancer, recovering from major surgery, or suffering from HIV infection. For such patients, antifungal therapy typically includes intravenous administration, of a formulation of one or more *P. syringae* lipodepsidecapeptides (e.g.,
20 the 25-B1 decapeptide antifungal agents) over several days to halt or retard the infection.

With respect to antifungal activity, the term "effective amount" means an amount of a compound of the present invention which is capable of inhibiting fungal growth or activity, or reducing symptoms of the fungal infection. For most fungal infections reduction of symptoms of the infection includes reduction of fever, return to
25 consciousness, and increased well being of the patient. Preferably, symptoms are reduced by killing the fungus to eliminate the infection or to bring the infection to a level tolerated by the patient or controlled by the patient's immune system. As used herein "inhibiting" refers to inhibiting fungal activity, including stopping, retarding or prophylactically hindering or preventing the growth or any attending characteristics and results from the
30 existence of a fungus.

Typically, the compositions will be administered to a patient (human or other animal, including mammals such as cats, horses and cattle and avian species) in need thereof, in an effective amount to inhibit the fungal infection. The dose administered will vary depending on such factors as the nature and severity of the infection, the age and
5 general health of the host and the tolerance of the host to the antifungal agent. The particular dose regimen likewise may vary according to such factors and may be given in a single daily dose or in multiple doses during the day. The regimen may last from about 2-3 days to about 2-3 weeks or longer. A typical daily dose (administered in single or divided doses) will contain a dosage level of from about 0.01 mg/kg to about 100 mg/kg
10 of body weight of an active compound of this invention. Preferred daily doses generally will be from about 0.1 mg/kg to about 60 mg/kg and ideally from about 2.5 mg/kg to about 40 mg/kg. For serious infections, the compound can be administered by intravenous infusion using, for example, 0.01 to 10 mg/kg/hr of the active ingredient.

The present invention also provides pharmaceutical formulations useful for
15 administering the antifungal compounds of the invention. Accordingly, the present invention also provides a pharmaceutical formulation including one or more pharmaceutically acceptable carriers, diluents, vehicles, excipients, or other additives and one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A. The active ingredient in such formulations includes from 0.1% to 99.9% by
20 weight of the formulation, more generally from about 10% to about 30% by weight. By "pharmaceutically acceptable" it is meant that the carrier, diluent or excipient is compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The formulation can include additives such as various oils, including those of
25 petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, and sesame oil. Suitable pharmaceutical excipients include starch, cellulose, glucose, lactose, sucrose, gelatin, malt, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, and ethanol. The compositions can be subjected to conventional pharmaceutical expedients.
30 such as sterilization, and can contain conventional pharmaceutical additives, such as

preservatives, stabilizing agents, wetting, or emulsifying agents, salts for adjusting osmotic pressure, and buffers. Suitable pharmaceutical carriers and their formulations are described in Martin, "Remington's Pharmaceutical Sciences," 15th Ed.; Mack Publishing Co., Easton (1975); see, e.g., pp. 1405-1412 and pp. 1461-1487.

5 The term "pharmaceutically acceptable salt", as used herein, refers to salts of the compounds of the above formula that are substantially non-toxic to living organisms. Typical pharmaceutically acceptable salts include those salts prepared by reaction of the compounds of the present invention with a mineral or organic acid or an inorganic base. Such salts are known as acid addition and base addition salts.

10 Acids commonly employed to form acid addition salts are mineral acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, and phosphoric acid, and organic acids such as p-toluenesulfonic, methanesulfonic acid, oxalic acid, p-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, and acetic acid. Examples of such pharmaceutically acceptable salts are the sulfate,
15 pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, isobutyrate, caproate, heptanoate, propiolate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate,
20 dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, sulfonate, xylenesulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, gamma-hydroxybutyrate, glycollate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, and mandelate. Preferred pharmaceutically acceptable acid addition salts are those formed with mineral acids such as hydrochloric
25 acid and hydrobromic acid, and those formed with organic acids such as maleic acid and methanesulfonic acid.

 Base addition salts include those derived from inorganic bases, such as ammonium or alkali or alkaline earth metal hydroxides, carbonates, and bicarbonates. Such bases useful in preparing the salts of this invention thus include sodium hydroxide, potassium
30 hydroxide, ammonium hydroxide, potassium carbonate, sodium carbonate, sodium

bicarbonate, potassium bicarbonate, calcium hydroxide, and calcium carbonate. The potassium and sodium salt forms are particularly preferred.

It should be recognized that the particular counterion forming a part of any salt of this invention is not of a critical nature, so long as the salt as a whole is pharmacologically acceptable and as long as the counterion does not contribute undesired qualities to the salt as a whole.

A *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, may be administered parenterally, for example using intramuscular, subcutaneous, or intra-peritoneal injection, nasal, or oral means. In addition to these methods of administration, a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, may be applied topically for superficial skin infections or to inhibit fungal growth in the mucus.

For parenteral administration the formulation includes one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, and a physiologically acceptable diluent such as deionized water, physiological saline, 5% dextrose and other commonly used diluents. The formulation may contain a cyclodextrin and/or a solubilizing agent such as a polyethylene glycol or polypropylene glycol or other known solubilizing agent. Such formulations may be made up in sterile vials containing the antifungal and excipient in a dry powder or lyophilized powder form. Prior to use, a physiologically acceptable diluent is added and the solution withdrawn via syringe for administration to the patient.

The present pharmaceutical formulations are prepared by known procedures using known and readily available ingredients. In making the compositions of the present invention, the active ingredient is generally admixed with a carrier, or diluted by a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet, paper or other container. When the carrier serves as a diluent, it may be a solid, semi-solid or liquid material which acts as a vehicle, excipient or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols, (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active

compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, or sterile packaged powders.

For oral administration, the antifungal compound is filled into gelatin capsules or formed into tablets. Such tablets may also contain a binding agent, a dispersant or other suitable excipients suitable for preparing a proper size tablet for the dosage and a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. For pediatric or geriatric use the antifungal compound may be formulated into a flavored liquid suspension, solution or emulsion. A preferred oral formulation is linoleic acid, cremophor RH-60 and water and preferably in the amount (by volume) of 8% linoleic acid, 5% cremophor RH-60, 87% sterile water and a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, in an amount of from about 2.5 to about 40 mg/ml.

For topical use the antifungal compound may be formulated with a dry powder for application to the skin surface or it may be formulated in a liquid formulation including a solubilizing aqueous liquid or non-aqueous liquid, e.g., an alcohol or glycol.

Uses of Formulations of a *P. syringae* Lipodepsidecapeptide

The present invention also encompasses a kit including the present pharmaceutical compositions and to be used with the methods of the present invention. The kit can contain a vial which contains a formulation of the present invention and suitable carriers, either dried or in liquid form. The kit further includes instructions in the form of a label on the vial and/or in the form of an insert included in a box in which the vial is packaged, for the use and administration of the compounds. The instructions can also be printed on the box in which the vial is packaged. The instructions contain information such as sufficient dosage and administration information so as to allow a worker in the field to administer the drug. It is anticipated that a worker in the field encompasses any doctor, nurse, or technician who might administer the drug.

The present invention also relates to a pharmaceutical composition including a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, and that is suitable for administration by injection. According to the

invention, a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be used for manufacturing a composition or medicament suitable for administration by injection. The invention also relates to methods for manufacturing compositions including a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, in a form that is suitable for oral or topical administration. For example, a liquid or solid formulation can be manufactured in several ways, using conventional techniques. A liquid formulation can be manufactured by dissolving the one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, in a suitable solvent, such as water, at an appropriate pH, including buffers or other excipients.

Agricultural Uses

Antibiotics produced from *P. syringae* NRRL B-12050 have been demonstrated to effectively treat Dutch elm disease. (see, e.g., U.S. Patent Nos. 4,342,746 and 4,277,462) In particular, *P. syringae* MSU 16H has been shown to confer a greater protection than the wild-type strain in elms infected with *Ceratocystis ulmi*, the causal agent of Dutch elm disease. (see e.g., Lam et al, *Proc. Natl. Sci. USA*, 84, 6447-6451 (1987)). More extensive tests on field-grown elms confirmed the phenomenon of biocontrol at the prophylactic level. Hence, the lipodepsidecapeptides of the present invention may be useful as a preventative treatment for Dutch Elm disease. The pseudomycins have been shown to be toxic to a broad range of plant-pathogenic fungi including *Rhynchosporium secalis*, *Ceratocystis ulmi*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Verticillium albo-atrum*, *Verticillium dahliae*, *Thielaviopsis basicola*, *Fusarium oxysporum* and *Fusarium culmorum*. (see Harrison, L., et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," *J. General Microbiology*, 7, 2857-2865 (1991).) Consequently, one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A (including hydrates, solvates, and esters thereof) may be useful in the treatment of fungi in plants (in particular, *V. albo-atrum*, *Rhizoctonia solani* and *F. oxysporum*) either as a direct treatment or preventative treatment. Generally, the infected plants are treated by injecting or spraying an aqueous suspension of the lipodepsidecapeptide compounds into

or onto the plant. Means of injection are well-known to those skilled in the art (e.g., gouge pistol). Any means of spraying the suspension may be used that distributes an effective amount of the active material onto the plant surface. The suspension may also include other additives generally used by those skilled in the art, such as solubilizers, stabilizers, wetting agents, and combinations thereof.

Treatment of the plant may also be accomplished using a dry composition containing one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A. The dry formulation may be applied to the plant surface by any means well-known to those skilled in the art, such as spraying or shaking from a container.

The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

EXAMPLES

Biological Materials on Deposit

P. syringae MSU 16H is publicly available from the American Type Culture Collection, Parklawn Drive, Rockville, MD, USA as Accession No. ATCC 67028. *P. syringae* strains 25-B1, 7H9-1, and 67 H1 were deposited with the American Type Culture Collection on March 23, 2000 and were assigned the following Accession Nos.:

| | |
|-------|------------------------|
| 25-B1 | Accession No. PTA-1622 |
| 7H9-1 | Accession No. PTA-1623 |
| 67 H1 | Accession No. PTA-1621 |

Example 1 – Production of 25-B1 Antifungal Agent A

Fermentation methods were developed for producing a lipodepsidecapeptide antifungal agent, 25-B1 decapeptide antifungal agent A, in the fermentation broth of a *Pseudomonas syringae* strain.

Materials and Methods

Preparation of inoculum: An aliquot of *P. syringae* strain 25-B1 cells stored in the vapor phase of liquid nitrogen was thawed and used to inoculate two 900 mL portions of CSM broth. CSM broth was composed of (g/L): dextrose (5), maltose (4), Difco Tryptic Soy Broth (30), Difco yeast extract (3), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2). Approximately 0.5 mL of cells was used to inoculate each 900 mL portion of medium contained in a two liter flask. Flasks were incubated with shaking for 24 hours at 25°C. The contents of two flasks were combined to inoculate a 150 liter fermentor containing 115 liters of sterile fermentation broth.

Fermentation Stage: Fermentation broth was composed of (g/L): dextrose (20), soluble starch (5), Basic American Foods Country Style Potato Pearls instant mashed potatoes (30), glycine (1), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), KCl (0.2), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.004) in tap water. The pH was adjusted to 5.2 before sterilization. Fermentation was carried out at 25°C for 68 hr. Dissolved oxygen was maintained at or above 30% of air saturation by continuous adjustment of air flow and impeller agitation rate. The pH was maintained between 4.0 and 5.4 through the addition of either H_2SO_4 or NaOH.

Several variations of the simple batch process were also found to produce the novel cyclic peptide product. Dextrose may be fed to the fermentors starting 24 hours after initial inoculation at a rate of 60 mL per hour. Feeding may be continued throughout the course of the fermentation. Alternatively, a process has been used where dissolved oxygen is maintained at 5% of air saturation starting 24 hours after inoculation and continuing until the end of the fermentation period. Maintenance of dissolved oxygen at 5% was achieved through addition of inert nitrogen gas (N_2) to the air supply leading to the fermentor. In all cases, gas was supplied through a single submerged sparger tube with an opening positioned just below the bottom agitator turbine in the fermentor.

Results and Conclusion

Several fermentation methods produce 25-B1 decapeptide antifungal agent A from *P. syringae*

Example 2 – Isolation and Purification of 25-B1 Antifungal Agent A

Methods were developed for isolation and purification of a lipodepsidecapeptide antifungal agent. 25-B1 decapeptide antifungal agent A. from the fermentation broth of a *Pseudomonas syringae* strain.

5

Materials and Methods

The whole fermentation broth produced according to Example 1, typically 100 L after harvest, was filtered through a Membralox™ ceramic filter (0.45 μ m). The resulting solid slurry was extracted with an equal volume of acetone containing 0.1% TFA for 90 min. The acetone extract was separated by filtration and evaporated *in vacuo* to an aqueous solution.

This solution was combined with the filtrate obtained from the ceramic filtration of the whole broth and charged on to an Amberchrom™ CG 300sd resin column (4 L) packed in water. The column was initially washed with 0.2% acetic acid (pH 4-8) until the effluent showed pH 4.5 followed by 10 L of 22% acetonitrile containing 0.2% acetic acid (pH 4.8). Then the column was eluted with a linear gradient of 22-35% acetonitrile containing 0.2% acetic acid (32 L) and 35% acetonitrile containing 0.2% acetic acid (8 L) with 400 ml/min flow rate. Fractions 11-16 were combined (4.8 L), concentrated *in vacuo* to 100 ml and centrifuged.

The supernatant was separated and chromatographed over an Amberchrom CG 300sd column (1 L) using a linear gradient of 25-35% acetonitrile containing 0.2% acetic acid (pH 4.8) with 50 ml/min flow rate. Fractions 20-25 (1.2 L) were combined and rechromatographed over a reversed-phase column (NovaPak C₁₈, 6 μ m, 40X300 mm, flow rate 40 ml/min, linear gradient 30-60% acetonitrile containing 0.2% TFA) to yield 21 mg of a compound (89% purity by UV).

The ESIMS data showed a possible [M+H]⁺ peak at m/z 1165.7, which is different from the known antifungal agents that have been found thus far from *P. Syringae*. The ¹H NMR spectrum showed signals reminiscent of pseudomycin-like lipopeptide but indicated the presence of more than one compound. In order to obtain 25-B1 decapeptide antifungal agent A in high purity for structure determination and antifungal activity, an

additional broth from 4X100 L fermentation was processed as described above and in addition, the final purification was carried out on a reversed-phase column [Rainin C₁₈, 6 μ m, 24X250 mm, 0.1% TFA-acetonitrile-MeOH (8:1:1 to 4:3:3) gradient elution for 60 min; (4:3:3 to 10:45:45) gradient elution for 30 min] to afford 42 mg of 25-B1
5 decapeptide antifungal agent A (93% purity by UV).

Results and Conclusion

HPLC methods similar to those used to purify other lipodepsi-peptide antifungal agents resulted in purification of 25-B1 decapeptide antifungal agent A from fermentation
10 broth.

Example 3 – Determination of the Structure of 25-B1 Antifungal Agent A

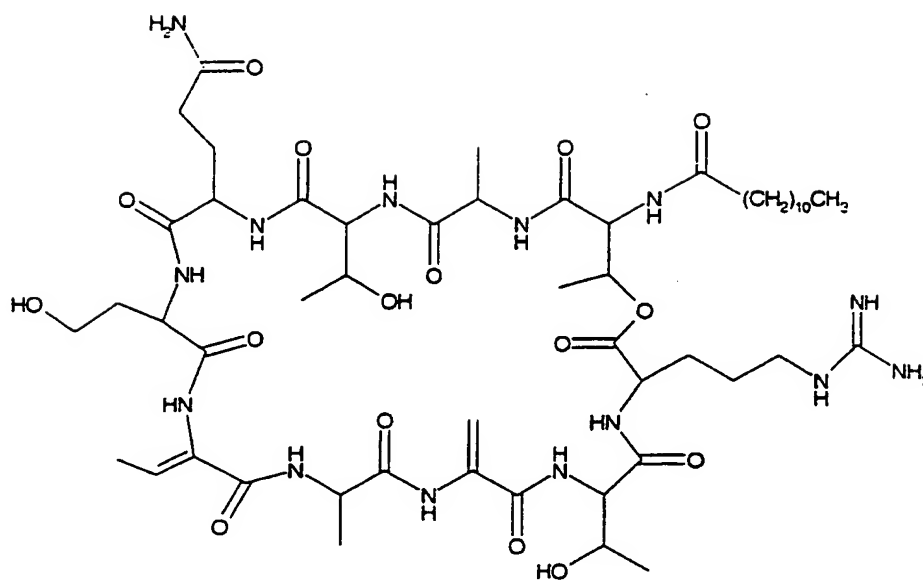
Mass spectrometry and NMR determined the structure of a lipodepsi-decapeptide antifungal agent, 25-B1 decapeptide antifungal agent A.
15

Methods and Results

The molecular formula of 25-B1 decapeptide antifungal agent A was determined by high resolution FABMS as C₅₂H₈₈N₁₄O₁₆ [m/z 1165.6581 for C₅₂H₈₉N₁₄O₁₆(M+H)⁺, Δ +0.9 ppm].
20

In accordance with this formula the ¹³C and DEPT NMR spectra showed 50 distinct resonances, which included twelve carbonyl carbons, five olefinic carbons, four oxygenated sp³ carbons, eight typical amino acid α -carbons, fifteen methylene carbons and six methyl carbons. Among these, one of the methyl carbon signals at δ 16.7 and one of the methylene carbon signals at δ 28.9 each constituted a set of degenerate carbons.
25 thus accounting for the total number of 52 carbons observed in the molecular formula.

Detailed analysis of ¹H, ¹³C, and 2D NMR (DQCOSY, TOCSY, HMQC, HMBC and ROESY) data enabled to determine the structure of 25-B1 decapeptide antifungal agent A (IA) and unambiguously assign all the protons and carbons (Table 1).



IA

Table 1. ^1H and ^{13}C NMR Chemical Shifts of 25-B1 decapeptide antifungal agent A in DMSO- d_6

| Amino acid | Position | δ_{H} | δ_{C} |
|------------|------------|---------------------|---------------------|
| Arg | NH | 8.13 | - |
| | CO | - | 170.4 |
| | α | 4.23 | 51.7 |
| | β_1 | 1.69 | 28.2 |
| | β_2 | 1.63 | |
| | γ | 1.46 | 24.7 |
| | δ | 3.08 | 40.2 |
| | NH | 7.59 | - |
| Thr-1 | ϵ | - | 156.7 |
| | NH | 7.83 | - |
| | CO | - | 169.8 |
| | α | 4.23 | 59.5 |
| | β | 4.00 | 66.4 |
| Dha | γ | 1.08 | 19.7 |
| | NH | 9.01 | - |
| | CO | - | 163.7 |
| | α | - | 135.3 |
| | β_1 | 5.86 | 106.4 |
| | β_2 | 5.61 | |

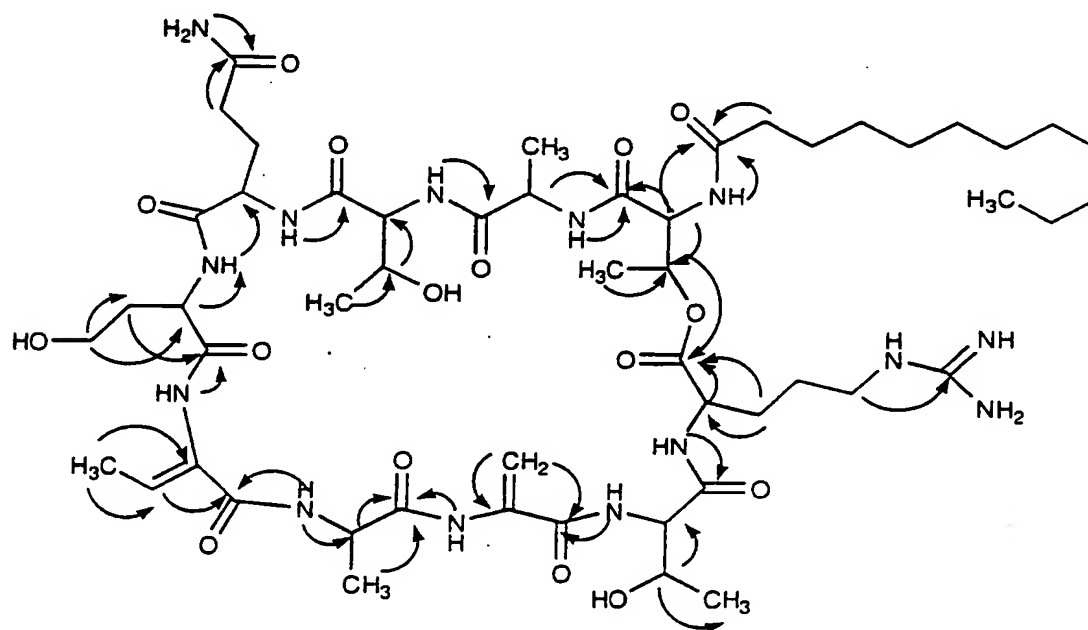
| Amino acid | Position | δ_H | δ_C |
|------------|-----------------|------------|------------|
| Ala-1 | NH | 7.96 | - |
| | CO | - | 171.6 |
| | α | 4.22 | 49.9 |
| | β | 1.31 | 16.7 |
| Dhb | NH | 9.07 | - |
| | CO | - | 164.0 |
| | α | - | 130.0 |
| | β | 6.40 | 128.5 |
| | γ | 1.61 | 12.9 |
| HSer | NH | 8.22 | - |
| | CO | - | 171.1 |
| | α | 4.22 | 51.4 |
| | β_1 | 1.82 | 34.0 |
| | β_2 | 1.76 | |
| | γ | 3.47 | 57.3 |
| Gln | NH | 7.59 | - |
| | CO | - | 171.9 |
| | α | 4.31 | 52.0 |
| | β_1 | 1.90 | 27.9 |
| | β_2 | 1.73 | |
| | γ | 2.11 | 31.1 |
| | CO | - | 173.9 |
| | NH ₂ | 7.13 | - |
| | | 6.72 | |
| Thr-2 | NH | 7.80 | - |
| | CO | - | 169.9 |
| | α | 4.11 | 58.4 |
| | β | 4.11 | 66.0 |
| | γ | 1.01 | 19.8 |
| Ala-2 | NH | 8.18 | - |
| | CO | - | 172.4 |
| | α | 4.32 | 48.5 |
| | β | 1.16 | 17.6 |

| Amino acid | Position | δ_H | δ_C |
|------------|----------|------------|--------------------------------------|
| Thr-3 | NH | 7.93 | - |
| | CO | - | 168.6 |
| | α | 4.47 | 55.6 |
| | β | 4.94 | 70.5 |
| | γ | 1.12 | 16.7 |
| | | | |
| Side chain | CO | | 172.2 |
| | 2 | 2.08 | 35.1 |
| | 3 | 1.44 | 25.1 |
| | 4 | 1.21 | 28.5 |
| | 5-10 | 1.21 | 31.1, 28.9X2, 28.8, 28.7, 28.6 |
| | 11 | 1.21 | 22.0 |
| | 12 | 0.83 | 13.9 |

The results from 1H , DQCOSY and TOCSY spectra measured in DMSO- d_6 at 35°C revealed the presence of spin systems for seven commonly occurring amino acid residues – two alanines, one arginine, one glutamine and three threonines, and three less commonly occurring amino acids – one dehydroalanine (Dha), one dehydroaminobutyric acid (Dhb) and one homoserine. The less commonly occurring amino acid residues, viz. Dha, Dhb and homoserine, were identified by the cross peaks observed in the TOCSY spectrum from the amide protons resonating at δ 9.01 (brs), 9.07 (brs) and 8.22 to protons resonating at δ 5.86, 5.61 (δ_C 106.4), 6.40 (δ_C 128.5), 1.61 (δ_C 12.9) and 4.22 (δ_C 51.4), 1.82, 1.76 (δ_C 34.0), 3.47 (δ_C 57.3), respectively. Consistent with this the ^{13}C NMR spectrum displayed eight proton bound α -carbon signals for the saturated amino acid residues and two quaternary α -carbons for the unsaturated amino acids Dha and Dhb. Of the twelve amide or ester type carbonyls, nine were assigned to the eight saturated amino acid residues (two to glutamine), two (δ 164.0 and 163.7 ppm) to Dha and Dhb. The remaining one carbonyl group was assigned to the dodecanoyl side chain, the presence of which is discerned from the terminal methyl signal (δ_H 0.83 and δ_C 13.9) and 10 methylene signals in the ^{13}C NMR spectrum (Table 1).

The molecular formula requires sixteen degrees of unsaturation. The ten amino acids and the dodecanoyl side chain accounted for fifteen sites of unsaturation indicating that 25-B1 decapeptide antifungal agent A is a monocyclic decapeptide. Comparison of the chemical shift of the β -protons of threonines (δ_H 4.94, 4.11 and 4.00) indicated that the proton resonating at δ 4.94 was attached to a carbinol which is modified to an ester or a lactone. That this was so and 25-B1 decapeptide antifungal agent A is a depsipeptide was evidenced by the HMBC and ROESY data. These data also established the amino acid sequence and the location of the dodecanoyl side chain in 25-B1 decapeptide antifungal agent A.

With amino acid Arg as a starting point, the long range $^1H - ^{13}C$ correlations observed in the HMBC spectrum between the amide proton and the adjacent amino acid carbonyl and or α -carbon (see Scheme I below) unambiguously established the amino acid sequence Arg-NH/Thr-CO, Thr-NH/Dha-CO, Dha-NH/Ala-CO, Ala-NH/Dhb-CO, Dhb-NH/Hse-CO, Hse-NH/Glu- α -C, Glu-NH/Thr-CO, Thr-NH/Ala CO and Ala-NH/Thr-CO.

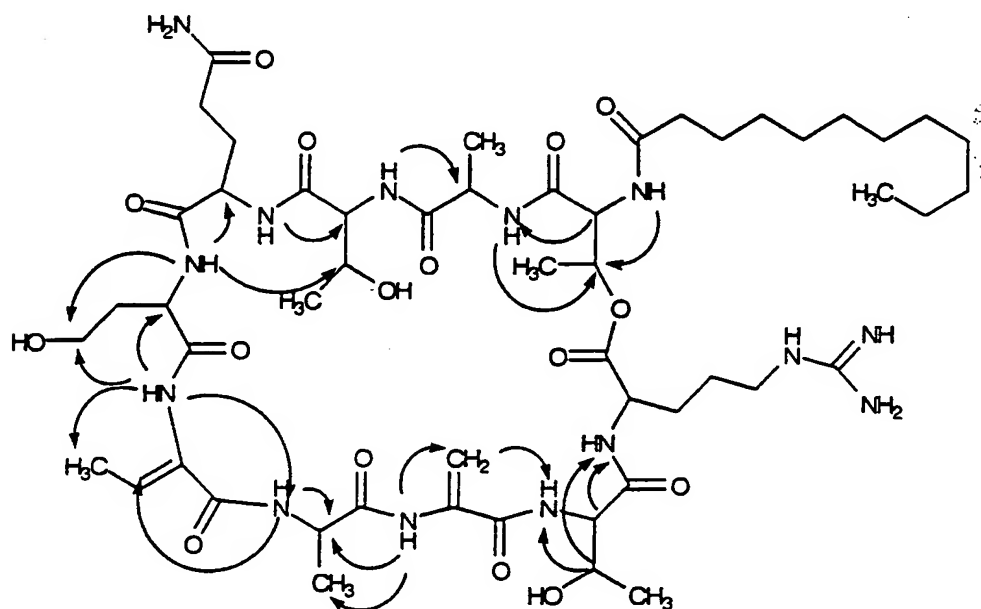


HMBC (H \rightarrow C) Correlations

Scheme I

The NH of Thr adjacent to Ala did not show a long range $^1\text{H} - ^{13}\text{C}$ correlation to the carbonyl of Arg residue, instead it showed a correlation to a carbonyl assigned to the dodecanoyl side chain. The absence of Thr-NH/Arg-CO correlation and presence of a correlation between the Thr- β -H (δ_{H} 4.94, δ_{C} 70.5)/Arg-CO clearly established an ester linkage between the Thr- β -OH and Arg-COOH. Consistent with these assignments are the ROESY correlations that were observed between the amide protons and the adjacent amino acid α -protons (see Scheme II below).

10



Selected ROESY Correlations

Scheme II

Conclusions

Compound 25-B1 decapeptide antifungal agent A represents a novel class of lipodepsipeptide which possesses several amino acid residues that are not present in any of the pseudomycins, syringomycins, syringotoxin and syringostatins produced by different isolates of *P. syringae*. The new depsipeptide is composed of ten amino acids

which is also a departure from the pseudomycins and syringomycins which possess only nine amino acid residues. Unlike the pseudomycin natural products, the new depsipeptide does not include chlorothreonine which is suspected to be the cause for irritation at the injection site of pharmaceutical formulations containing pseudomycin compounds.

5

Example 4 -- Antifungal activity of 25-B1 Decapeptide Antifungal Agent A

The antifungal studies were conducted using a microtiter broth dilution assay according to National Committee for Clinical Laboratory Standards guidelines in 96 well microtiter plates. Sabourauds and dextrose broth was adjusted to contain 2.5×10^4 conida/ml. Test compound was dissolved in water and tested in two-fold dilutions starting with the highest concentration of 20 $\mu\text{g/ml}$. Plates were incubated at 35°C for 48 hr. The results in Table 2 show the minimal inhibitory concentration (MIC) of the compound that completely inhibited growth compared to untreated growth controls.

15

Table 2. Antifungal activity of 1

| Organism | MIC ($\mu\text{g/ml}$) |
|--------------------------------|--------------------------|
| <i>Candida albicans</i> | 10 |
| <i>C. parapsilosis</i> | 10 |
| <i>Cryptococcus neoformans</i> | 1.25 |
| <i>Aspergillus fumigatus</i> | >20 |
| <i>Histoplasma capsulatum</i> | 20 |

The presence or amount of one or more *P. syringae* lipodepsipeptides, such as 25-B1 decapeptide antifungal agent A, can be determined by measuring the antifungal activity of a preparation. Antifungal activity can be determined in vitro by obtaining the minimum inhibitory concentration (MIC) of the preparation using a standard agar dilution test or a disc diffusion test. A preparation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be an extract of a cell culture, or a more purified mixture. A typical fungus employed in testing antifungal activity is *C. albicans*. Antifungal activity is considered significant when the test preparation causes 10-12 mm diameter zones of inhibition on *Candida albicans* x657 seeded agar plates.

Example 5 -- Isolation, Characterization and Mutagenesis of *Pseudomonas syringae*

Environmental isolates and mutants of *P. syringae* were produced and employed in production of antifungal agents.

Materials and Methods

Strains MSU 174 and MSU 16-H were isolated and characterized as described in U.S. Patent No. 5,576,298, issued November 19, 1996 to G. Strobel et al.; Harrison et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," J. Gen. Microbiology **137**, 2857-2865 (1991); and Lamb et al., "Transposon mutagenesis and tagging of fluorescent pseudomonas: Antimycotic production is necessary for control of Dutch elm disease," Proc. Natl. Acad. Sci. USA **84**, 6447-6451 (1987). The disclosures of the references cited in this paragraph are incorporated herein by reference.

Additional strains were derived from such wild type and transposon generated mutants by chemical mutagenesis. Strains subjected to mutagenesis include MSU 174, MSU 16H, and 25-B1. The strain to be mutagenized was grown in a medium containing potato product, then divided into the medium including 0, 1, 2, 4, 16, or 32 μ M of the chemical mutagen 1-methyl-3-nitro-1-nitrosoguanidine (NTG or MNNG). These cells were then frozen for future screening and selection.

Mutagenized cells were selected for desirable growth conditions and/or production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A. Chemically mutagenized cells of *P. syringae*, such as mutagenized strain 25-B1, were thawed and diluted to 6 cells/mL in N21SM medium (Table 3). This medium sometimes contained one or more components for selection, such as varying concentrations of phosphate. A 50 μ L volume of mutagenized cells was dispensed into a well of a 96-well round bottom microtiter plate for a delivery of an average of 0.3 cells/well. Typically, silicone oil was added to each well to minimize evaporation. The plates were incubated with shaking for 6 to 12 days at 25 °C.

Table 3 -- The Composition of N21SM Medium

| INGREDIENT | GRAMS PER LITER |
|----------------------------------|--------------------|
| Glucose | 20 |
| Ammonium Sulfate | 0.5 |
| Monosodium Glutamate | 2 |
| L-Histidine | 2 |
| Glycine | 0.5 |
| Soluble Starch | 5 |
| KH ₂ PO ₄ | 0.2 |
| Czapek Mineral Salts Solution | 2 mL |
| MES Buffer | 9.8 |
| Adjust pH to 5.0 | |

After this incubation, an aliquot, typically 5 μ L, from each well was serially diluted (e.g. 1:56, 1:196, 1:320, 1:686, and/or 1:1715) and evaluated for activity against *Candida albicans* in a liquid microtiter plate bioassay. The plates were incubated at 37 °C overnight and the wells were scored for inhibition of *C. albicans* growth. Suitable strains were picked, inoculated into CSM medium (Table 4), and grown for 1 to 3 days at 25 °C.

Table 4. Complete Streptomyces Medium (CSM)

| Component | Concentration (g/L) |
|-------------------------------------|---------------------|
| Glucose | 5 |
| Maltose | 4 |
| Difco Tryptic Soy Broth | 30 |
| Difco Yeast Extract | |
| MgSO ₄ 7H ₂ O | 2 |
| No pH adjustment | |

The selected strains were preserved and inoculated into fermentation bottles containing 13mL of N21SM medium and grown for approximately 66 hours at 25 °C. An aliquots was removed from this fermentation, extracted for 1 hour with a volume of acetonitrile equal to the volume of the aliquot, centrifuged, and decanted for HPLC analysis of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, as described in Examples 1-3. Strains producing one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, were reisolated, refermented, and prepared for growth on a larger scale.

Results

Strains exhibiting production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, were produced using the methods described above.

Conclusion

The selection methods and criteria disclosed herein are effective for producing strains of *P. syringae* that grow on minimal medium and produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A.

Example 6 – Formulations Including *P. syringae* Lipodepsidecapeptide

The following formulation examples are illustrative only and are not intended to limit the scope of the invention in any way. The term "active ingredient" means a *P. syringae* lipodepsidecapeptide or a pharmaceutically acceptable salt thereof.

5

Formulation 1

Hard gelatin capsules are prepared using the following ingredients:

| Ingredient | Quantity (mg/capsule) |
|--------------------|--------------------------|
| Active ingredient | 250 |
| Starch, dried | 200 |
| Magnesium stearate | 10 |
| Total | 460 mg |

10 **Formulation 2**

A tablet is prepared using the ingredients below. The components are blended and compressed to form tablets each weighing 665 mg.

| Ingredient | Quantity (mg/capsule) |
|-----------------------------|--------------------------|
| Active ingredient | 250 |
| Cellulose, microcrystalline | 400 |
| Silicon dioxide, fumed | 10 |
| Stearic acid | 5 |
| Total | 665 mg |

15 **Formulation 3**

An aerosol solution is prepared containing the following components. The active compound is mixed with ethanol and the mixture added to a portion of the propellant 22, cooled to – 30° C. and transferred to a filling device. The required amount is then fed to a stainless steel container and diluted with the remainder of the propellant. The valve units

20 are then fitted to the container.

| Component | Weight (g) |
|--|------------|
| Active ingredient | 0.25 |
| Methanol | 27.75 |
| Propellant 22 (Chlorodifluoromethane) | 74.00 |
| Total | 100.00 |

Formulation 4

Tablets, each containing 60 mg of active ingredient, are made as follows:

5

| | |
|--|--------|
| Active ingredient | 60 mg |
| Microcrystalline cellulose | 45 mg |
| Polyvinylpyrrolidone (as 10% solution in water) | 4 mg |
| Sodium carboxymethyl starch | 4.5 mg |
| Magnesium stearate | 0.5 mg |
| Talc | 1 mg |
| Total | 150 mg |

The active ingredient, starch and cellulose are passed through a No. 45 mesh U.S. sieve and mixed thoroughly. The aqueous solution containing polyvinyl-pyrrolidone is mixed with the resultant powder, and the mixture then is passed through a No. 14 mesh U.S. sieve. The granules so produced are dried at 50° C. and passed through a No. 18 mesh U.S. sieve. The sodium carboxymethyl starch, magnesium stearate and talc, previously passed through a No. 60 mesh U.S. sieve, are then added to the granules which, after mixing, are compressed on a tablet machine to yield tablets each weighing 150 mg.

15

Formulation 5

Capsules, each containing 80 mg of active ingredient, are made as follows:

| | |
|----------------------------|--------|
| Active ingredient | 80 mg |
| Starch | 59 mg |
| Microcrystalline cellulose | 59 mg |
| Magnesium stearate | 2 mg |
| Total | 200 mg |

The active ingredient, cellulose, starch and magnesium stearate are blended, passed through a No. 45 mesh U.S. sieve, and filled into hard gelatin capsules in 200 mg quantities.

5

Formulation 6

Suppositories, each containing 225 mg of active ingredient, are made as follows:

| | |
|---------------------------------|----------|
| Active ingredient | 225 mg |
| Saturated fatty acid glycerides | 2,000 mg |
| Total | 2,225 mg |

10

The active ingredient is passed through a No. 60 mesh U.S. sieve and suspended in the saturated fatty acid glycerides previously melted using the minimum heat necessary. The mixture is then poured into a suppository mold of nominal 2 g capacity and allowed to cool.

15

Formulation 7

Suspensions, each containing 50 mg of active ingredient per 5 ml dose, are made as follows:

| | |
|--------------------------------|---------|
| Active ingredient | 50 mg |
| Sodium carboxymethyl cellulose | 50 mg |
| Syrup | 1.25 ml |
| Benzoic acid solution | 0.10 ml |
| Flavor | q.v. |
| Color | q.v. |
| Purified water to total | 5 ml |

The active ingredient is passed through a No. 45 mesh U.S. sieve and mixed with the sodium carboxymethyl cellulose and syrup to form a smooth paste. The benzoic acid solution, flavor and color are diluted with a portion of the water and added, with stirring. Sufficient water is then added to produce the required volume.

5

Formulation 8

An intravenous formulation may be prepared as follows. The solution of these ingredients generally is administered intravenously to a subject at a rate of 1 ml per minute.

| | |
|-------------------|----------|
| Active ingredient | 100 mg |
| Isotonic saline | 1,000 mg |

10

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

WE CLAIM:

1. An isolated *P. syringae* depsidecapeptide, or a pharmaceutically acceptable salt, ester, or hydrate thereof, comprising a depsidecapeptide ring wherein the depsidecapeptide ring comprises arginine, threonine, homoserine, dehydroaminobutyric acid, and dehydroalanine, and a lactone is formed from a carboxyl group of the arginine and a hydroxyl group of the threonine.

2. The *P. syringae* depsidecapeptide of claim 1, wherein the depsidecapeptide ring has a sequence:
Thr -Ala-Thr-Gln-Hse-Dhb-Ala-Dha-Thr-Arg (SEQ ID NO: 1).

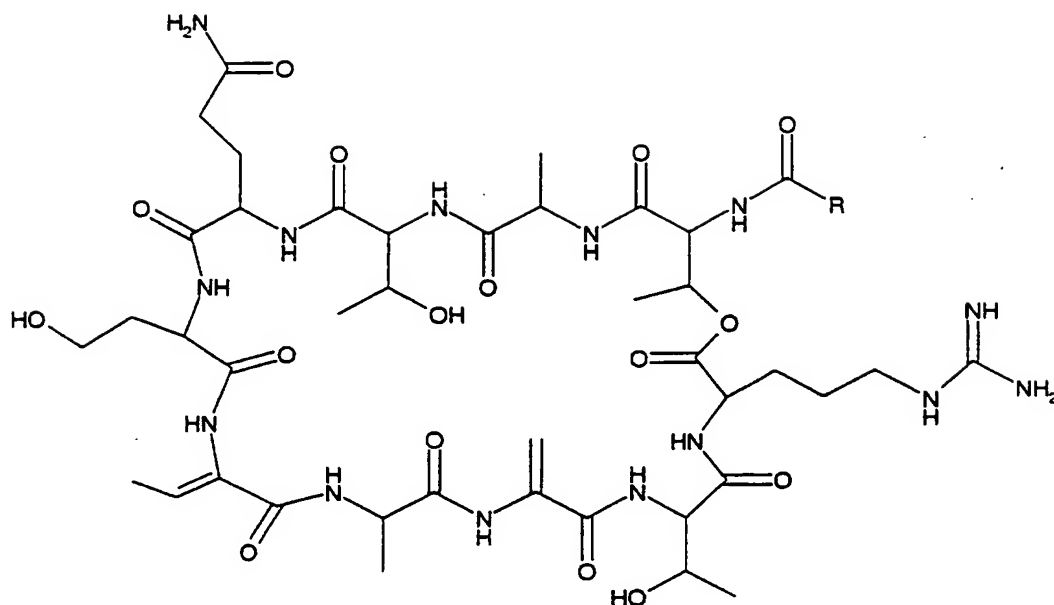
3. The *P. syringae* depsidecapeptide of claim 1, wherein the *P. syringae* depsidecapeptide is a *P. syringae* lipodepsidecapeptide.

4. The *P. syringae* depsidecapeptide of claim 3, wherein the *P. syringae* lipodepsidecapeptide comprises a fatty acid moiety coupled to an amino group of the threonine by an amide bond.

5. The *P. syringae* depsidecapeptide of claim 4, wherein the fatty acid moiety is a decanoic acid moiety, a decanoic acid moiety substituted with one or two hydroxyl groups, a dodecanoic acid moiety, a dodecanoic acid moiety substituted with one or two hydroxyl groups, a tetradecanoic acid moiety, or a tetradecanoic acid moiety substituted with one or two hydroxyl groups.

6. The *P. syringae* depsidecapeptide of claim 5, wherein the fatty acid moiety is an *n*-dodecanoic acid moiety.

7. The *P. syringae* depsidecapeptide of claim 3, wherein the *P. syringae* lipodepsidecapeptide is represented by the formula:



where R is a lipophilic moiety, or a pharmaceutically acceptable salt, ester, or hydrate thereof.

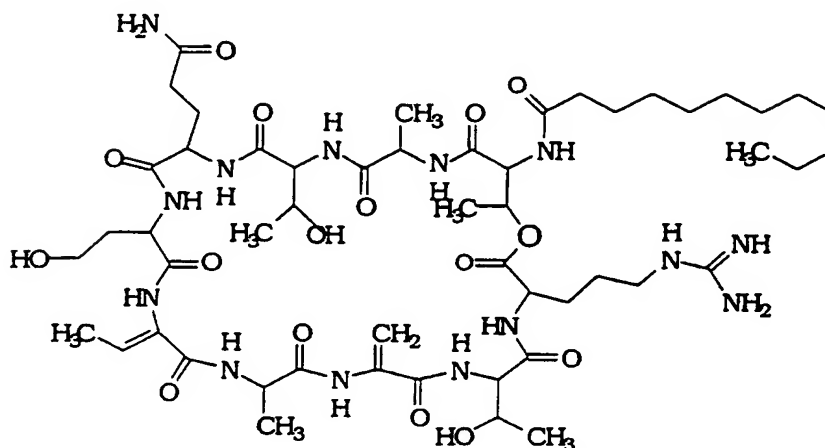
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8. The *P. syringae* depsidecapeptide of claim 7, wherein the lipophilic moiety is selected from the group consisting of C₉-C₁₅ alkyl, C₉-C₁₅ hydroxyalkyl, C₉-C₁₅ dihydroxyalkyl, C₉-C₁₅ alkenyl, C₉-C₁₅ hydroxyalkenyl, and C₉-C₁₅ dihydroxyalkenyl.

10

9. The *P. syringae* depsidecapeptide of claims 8, wherein the lipophilic moiety is C₉-C₁₅ alkyl.

10. An isolated *P. syringae* depsidecapeptide having the formula:



- 5 or a pharmaceutically acceptable salt, ester, or hydrate thereof.

11. A method of inhibiting fungal activity comprising contacting a fungus with an isolated *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

- 10 12. The method of claim 11, wherein the fungus comprises *Candida parapsilosis*, *Candida albicans*, *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

13. A method of reducing the symptoms of a fungal infection in a patient in need thereof comprising:

- 15 administering to the patient an effective amount of a composition comprising an isolated *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

14. The method of claim 13, wherein reducing the symptoms comprises decreasing a burden of a fungal infection.

15. The method of claim 13, wherein the fungal infection comprises infection by *Candida parapsilosis*, *Candida albicans*, *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

5 16. The method of claim 13, further comprising the steps of:
determining the need for administering isolated *P. syringae* depositeptide; and
monitoring the patient for relief of symptoms of the fungal infection.

10 17. The method of claim 13, wherein administering comprises parenteral
administration about 1 to about 3 times per day of about 0.1 to about 5 mg/kg of isolated
P. syringae depositeptide.

18. The method of claim 13, wherein reducing the symptoms of a fungal
infection comprises reducing fever and increasing general well being of the patient.

15 19. Use of an isolated *P. syringae* depositeptide of Claims 1, 2, 3, 4, 5, 6,
7, 8, 9 or 10 in the manufacture of a medicament for the treatment of a fungal infection.

20 20. The use of Claim 19 wherein the fungal infection comprises infection by
Candida parapsilosis, *Candida albicans*, *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

21. A method for producing one or more *P. syringae* depositeptides
comprising the steps of:
25 culturing a biologically pure culture of *Pseudomonas syringae* in nutrient medium
comprising three or fewer amino acids at a pH of about 4 to about 6.5 until one or more *P.*
syringae depositeptide is produced at a concentration of at least about 10 µg/mL; and
recovering one or more *P. syringae* depositeptides, or a pharmaceutically
acceptable salt, ester, or hydrate thereof, from the culture.

30

22. The method of claim 21, wherein the amino acid comprises glutamic acid, glycine, histidine, or a combination thereof.

23. The method of claim 22, wherein the amino acid comprises glycine.

24. The method of claim 22, wherein the nutrient medium further comprises soluble starch, yeast extract, or a combination thereof and a lipid.

25. The method of claim 24, wherein the nutrient medium comprises a potato product and a lipid selected from the group consisting of soybean oil, fatty acids, and fatty acid esters.

26. The method of claim 25, wherein the potato product comprises potato dextrose broth, mashed potato mix, potato dextrin, potato protein, or a combination thereof.

27. The method of claim 21, wherein during the culturing step the dissolved oxygen concentration is maintained at about 5% to 30%.

28. The method of claim 21, wherein the culturing step further comprises feeding of glucose, ammonium hydroxide, or a combination thereof.

29. The method of claim 21, wherein the *P. syringae* depsidecapeptide produced is a *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

30. The method of claim 21, wherein the *Pseudomonas syringae* comprises a strain derived from strain MSU 16H, MSU 174, or MSU 206.

31. The method of claim 21, wherein the *Pseudomonas syringae* comprises strain MSU 16H, strain 25-B1, strain 67H1, or strain 7H9-1.

32. The method of claim 31, wherein the *Pseudomonas syringae* comprises strain 25-B1.

5 33. A *P. syringae* depsidecapeptide of Claim 7 prepared by the methods of Claims 21, 22, 23, 24, 25, 26, 27, 28, 30, 31 or 32.

34. A method for treating or preventing fungal growth in a plant comprising contacting a fungus with a *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9
10 or 10.

35. The method of Claim 33 wherein the fungus is *Rynchosporium secalis*, *Ceratocystis ulmi*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Verticillium albo-atrum*, *Verticillium dahliae*, *Thielaviopsis basicola*, *Fusarium oxysporum* or *Fusarium culmorum*.

15

36. The method of Claim 34 wherein the fungus is *V. albo-atrum*, *Rhizoctonia solani* or *F. oxysporum*.

SEQUENCE LISTING

<110> Eli Lilly and Company

<120> Antifungal Agents Isolated from Pseudomonas Syringae

<130> X-11015 Sequence Listing

<140>

<141>

<150> 60/129,446

<151> 1999-04-15

<160> 1

<170> PatentIn Ver. 2.1

<210> 1

<211> 10

<212> PRT

<213> Pseudomonas syringae

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<223> Xaa at position 5 is homoserine.

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<223> Xaa at position 6 is dehydroaminobutyric acid.

<220>

<223> Xaa at position 8 is dehydroalanine.

<400> 1

Thr Ala Thr Gln Xaa Xaa Ala Xaa Thr Arg

1

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X-11015

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| | | |
|-------|--|---|
| 0-1 | Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis) | |
| 0-1-1 | Prepared using | PCT-EASY Version 2.90 (updated 08.03.2000) |
| 0-2 | International Application No. | |
| 0-3 | Applicant's or agent's file reference | X-11015 |
| 1 | The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on: | |
| 1-1 | page | 19 |
| 1-2 | line | 20 |
| 1-3 | Identification of Deposit | |
| 1-3-1 | Name of depositary institution | American Type Culture Collection |
| 1-3-2 | Address of depositary institution | 10801 University Blvd., Manassas, Virginia 20110-2209 United States of America |
| 1-3-3 | Date of deposit | 23 March 1999 (23.03.1999) |
| 1-3-4 | Accession Number | ATCC PTA-1622 |
| 1-4 | Additional Indications | NONE |
| 1-5 | Designated States for Which Indications are Made | all designated States |
| 1-6 | Separate Furnishing of Indications These indications will be submitted to the International Bureau later | NONE |
| 2 | The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on: | |
| 2-1 | page | 19 |
| 2-2 | line | 21 |
| 2-3 | Identification of Deposit | |
| 2-3-1 | Name of depositary institution | American Type Culture Collection |
| 2-3-2 | Address of depositary institution | 10801 University Blvd., Manassas, Virginia 20110-2209 United States of America |
| 2-3-3 | Date of deposit | 23 March 2000 (23.03.2000) |
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| 2-4 | Additional Indications | NONE |
| 2-5 | Designated States for Which Indications are Made | all designated States |
| 2-6 | Separate Furnishing of Indications These indications will be submitted to the International Bureau later | NONE |

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X-11015

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| | | |
|------------|---|--|
| 3 | The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on: | |
| 3-1 | page | 19 |
| 3-2 | line | 22 |
| 3-3 | Identification of Deposit | |
| 3-3-1 | Name of depositary institution | American Type Culture Collection |
| 3-3-2 | Address of depositary institution | 10801 University Blvd., Manassas, Virginia 20110-2209 United States of America |
| 3-3-3 | Date of deposit | 23 March 2000 (23.03.2000) |
| 3-3-4 | Accession Number | ATCC PTA-1621 |
| 3-4 | Additional Indications | NONE |
| 3-5 | Designated States for Which Indications are Made | all designated States |
| 3-6 | Separate Furnishing of Indications | NONE |
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/08724

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K11/02 A61K38/15 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| A | US 4 416 874 A (KAPLAN ET AL.) 22 November 1983 (1983-11-22) the whole document | 1-36 |
| A | WO 95 11310 A (CHEIL FOODS AND CHEMICALS INC.) 27 April 1995 (1995-04-27) the whole document | 1-36 |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Masturzo, P

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/08724

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
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| WO 9511310 A | 27-04-1995 | KR 9709888 B | 19-06-1997 |
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| | | EP 0724645 A | 07-08-1996 |
| | | JP 10500098 T | 06-01-1998 |
| | | US 5786460 A | 28-07-1998 |

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(21) International Application Number: PCT/US00/08724

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ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,
LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ,
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(71) Applicant (for all designated States except US): ELI
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(84) Designated States (regional): ARIPO patent (GH, GM,
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(AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent
(AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU,
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(71) Applicants and

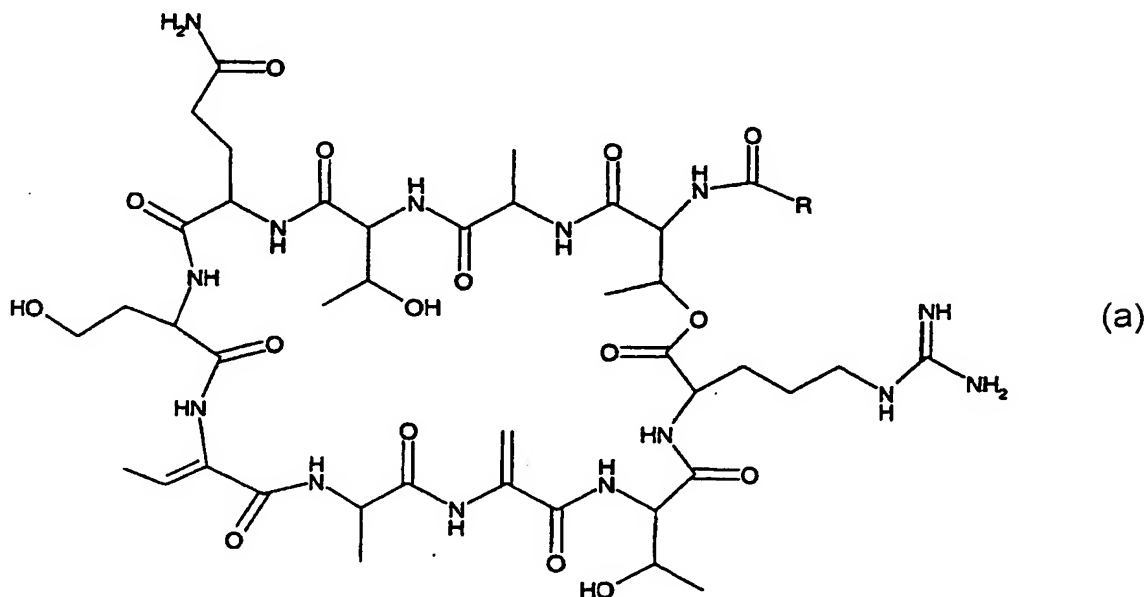
(72) Inventors: KULANTHAIVEL, Palaniappan [US/US];
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field, IN 46140 (US). MARTIN, James, William
[US/US]; 177 Mill Springs, Coatesville, IN 46121 (US).

Published:

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[Continued on next page]

(54) Title: ANTIFUNGAL AGENTS ISOLATED FROM *PSEUDOMONAS SYRINGAE*



(57) Abstract: The invention relates to *P. syringae* depsidecapeptides, method for making such peptide, and methods employing an-
tifungal activity of these peptides. The *P. syringae* depsidecapeptides include a compound having formula (a) where R is a lipophilic
moiety, or a pharmaceutically acceptable salt, ester, or hydrate thereof.



— *With (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description.*

(15) Information about Correction:

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ANTIFUNGAL AGENTS ISOLATED FROM *PSEUDOMONAS SYRINGAE*

5

FIELD OF THE INVENTION

The present invention relates to *P. syringae* depsidecapeptides, method for making such peptide, and methods employing antifungal activity of these peptides.

10

BACKGROUND

Fungal infections are a significant cause of disease, degradation of quality of life, and mortality among humans, particularly for immune compromised patients. The incidence in fungal infections in humans has increased greatly in the past 20 years. This is in part due to increased numbers of people with immune systems weakened or devastated by organ transplants, cancer chemotherapy, AIDS, age, and other similar disorders or conditions. Such patients are prone to attack by fungal pathogens that are prevalent throughout the population but are kept in check by a functioning immune system. These pathogens are difficult to control because some existing antifungal agents are either highly toxic or only inhibit fungal activity. For example, the polyenes are fungicidal but toxic; whereas, the azoles are much less toxic but only fungistatic. More importantly, there have been recent reports of azole and polyene resistant strains of *Candida* which severely limits therapy options against such strains.

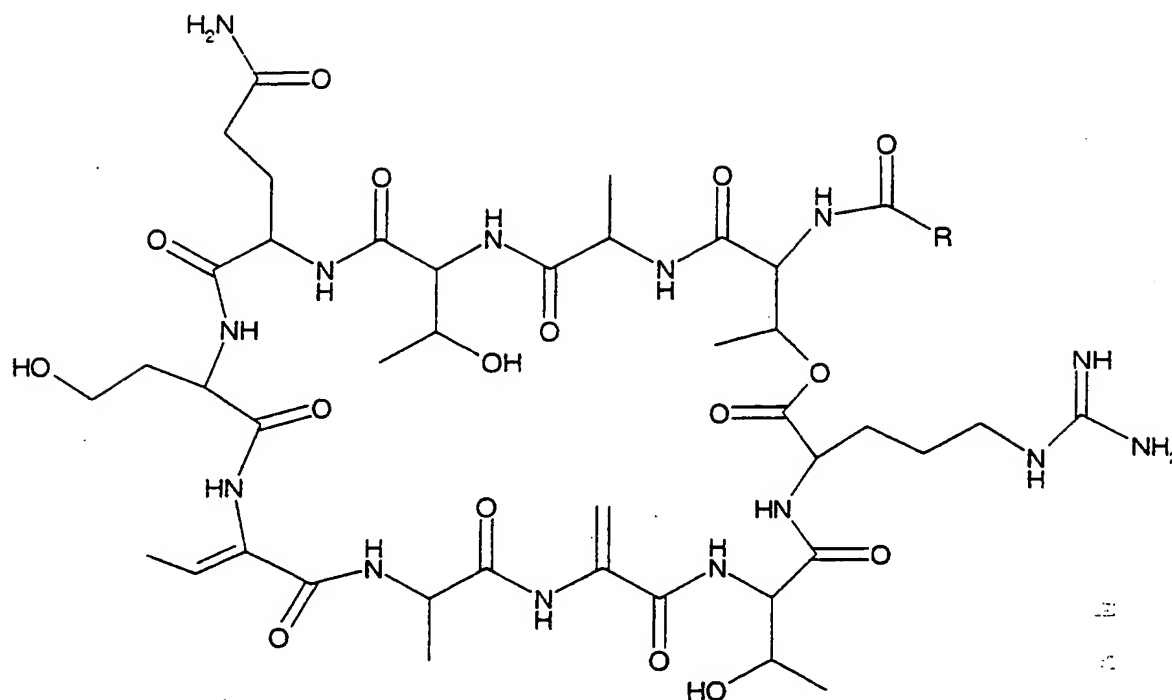
Pseudomonas syringae produce several classes of antifungal or antibiotic agents, such as the pseudomycins, syringomycins, syringotoxins, and syringostatins, which are lipodepsinonapeptides. Natural strains and transposon generated mutants of *P. syringae* produce these lipodepsinonapeptides. Several of the pseudomycins, syringomycins and other lipodepsipeptide antifungal agents have been isolated, chemically characterized, and shown to possess wide spectrum antifungal activity, including activity against important fungal pathogens in both humans and plants. The pseudomycins, the syringomycins, the

syringotoxins, and the syringostatins represent structurally distinct families of antifungal compounds.

None of the *P. syringae* lipodepsinonapeptides has been brought to market for antifungal therapy. Discovery of undesirable side effects, making formulations, scaling up production, and other development problems have thus far prevented exploitation of the *P. syringae* lipodepsinonapeptides against the full range of fungal infections that affect animals, humans and plants. There remains a need for an antifungal agent that can be used against infections not treated by existing antifungal agents and for application against infections in animals, humans, or plants.

SUMMARY OF THE INVENTION

The present invention provides a depsidecapeptide produced by *P. syringae* which contains the unusual amino acids homoserine (Hse), dehydroaminobutyric acid (Dhb) and dehydroalanine (Dha) as part of a depsidecapeptide ring. More specifically, the *P. syringae* depsidecapeptide includes a depsidecapeptide ring having the amino acids, arginine, threonine, homoserine, dehydroaminobutyric acid, and dehydroalanine, and a lactone formed from a carboxyl group of the arginine and a hydroxyl group of the threonine. As isolated from *P. syringae*, the depsidecapeptide is a lipodepsidecapeptide: a cyclic peptide coupled to a lipophilic moiety. Typically the lipophilic moiety is a fatty acid moiety coupled to the amino group of the threonine by an amide bond. Preferably, the fatty acid moiety is an *n*-dodecanoic acid moiety. The lipodepsidecapeptide is represented by formula I:



I

where R is a lipophilic moiety. The lipophilic moiety includes C₉-C₁₅ alkyl, C₉-C₁₅
 5 hydroxyalkyl, C₉-C₁₅ dihydroxyalkyl, C₉-C₁₅ alkenyl, C₉-C₁₅ hydroxyalkenyl, or C₉-C₁₅
 dihydroxyalkenyl. Preferably, the lipophilic moiety is C₁₁ alkyl. The alkyl, hydroxyalkyl,
 dihydroxyalkyl, alkenyl, hydroxyalkenyl, or dihydroxyalkenyl groups may be branched or
 unbranched. Preferably, the amino acid sequence of the depsidecapeptide ring is
 threonine-alanine-threonine-glutamine-homoserine-dehydroaminobutyric acid-alanine-
 10 dehydroalanine-threonine-arginine, referred to herein as "25-B1 decapeptide" or
 "Thr-Ala-Thr-Gln-Xaa-Xaa-Ala-Xaa-Thr-Arg (SEQ ID NO: 1)".

As used herein, the term "25-B1 decapeptide antifungal agent A" refers to the specific
 depsidecapeptide having the preferred amino acid sequence SEQ ID NO: 1 and R =
 unbranched C₁₁ alkyl (i.e., R = -(CH₂)₁₀CH₃).

15 The invention also relates to methods employing a *P. syringae* depsidecapeptide
 for inhibiting fungal activity or for reducing the symptoms of a fungal infection in a
 patient in need thereof. Such methods can kill the fungus, decrease the burden of a fungal

infection, reduce fever and increase general well being of a patient. Consequently, the *P. syringae* depsidecapeptides may be used in the manufacture of a medicament for treatment of a patient as described herein. The methods and medicaments of the invention are effective against fungi such as *Candida parapsilosis*, *Candida albicans*,
5 *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

The invention provides using microorganisms in a method for producing an anti-fungal agent, such as the *P. syringae* depsidecapeptides described above and including a 25-B1 decapeptide. The method involves culturing *Pseudomonas syringae* in media including three or fewer amino acids and recovering one or more *P. syringae*
10 depsidecapeptides from the culture. In one embodiment, *P. syringae* culture is in medium including glycine and a lipid, a potato product, or a combination thereof at a pH of about 4 to 6.5 until one or more *P. syringae* depsidecapeptides is produced at a concentration of at least about 10 $\mu\text{g/mL}$. In addition, the invention provides *P. syringae* depsidecapeptides prepared by the method described above.

15 The invention also provides a method for treating or preventing fungal growth in a plant whereby a fungus is contacted with a one or more of the *P. syringae* depsidecapeptides described above.

DETAILED DESCRIPTION

20 Lipodepsidecapeptide Antifungal Agents

As used herein "lipodepsidecapeptide antifungal agent" refers to an antifungal agent having a cyclic decapeptide ring closed by a lactone group and having an appended hydrophobic group, such as a fatty acid moiety. Lipodepsidecapeptide antifungal agents are produced by *Pseudomonas syringae*. A representative of this class of compounds, 25-B1 decapeptide antifungal agent A, has been purified and its structure determined. As
25 used herein the term "*P. syringae* lipodepsidecapeptide" refers to a lipodepsidecapeptide antifungal agent produced by *P. syringae*, and includes 25-B1 decapeptide antifungal agent A and related analogs.

P. syringae lipodepsidecapeptides share several structural features. For example,
30 each of these antifungal agents includes the unusual amino acids homoserine (Hse),

dehydroaminobutyric acid (Dhb) and dehydroalanine (Dha) as part of a depsidecapeptide ring. In each of the *P. syringae* lipodepsidecapeptides, a carboxyl group of an arginine residue linked to the hydroxyl group of the N-terminal threonine forms a lactone that closes the depsidecapeptide ring. The sequence of the depsidecapeptide ring of the *P.*

5 *syringae* lipodepsidecapeptide can be represented as:

Thr-Xaa-Xbb-Xcc-Hse-Dhb-Xdd-Dha-Xee-Arg

in which each of Xaa, Xbb, Xcc, Xdd, and Xee are individually naturally occurring amino acids. Unlike the pseudomycin natural products, the lipodepsidecapeptides of the present invention do not contain chlorothreonine which is suspected to be the cause for irritation
10 at the injection site of pharmaceutical formulations containing pseudomycin compounds.

The depsidecapeptide ring is linked to a lipophilic moiety, such as a fatty acid, through an amide bond with an amino group of the N-terminal threonine. The fatty acid generally includes 10, 12, 14, or 16 carbons, typically bearing zero, one or two hydroxyl groups. The fatty acid may be branched or unbranched and may also contain at least one
15 unsaturation. Preferred fatty acid moieties include an *n*-decanoic acid moiety, an *n*-decanoic acid moiety substituted with one or two hydroxyl groups, an *n*-dodecanoic acid moiety, an *n*-dodecanoic acid moiety substituted with one or two hydroxyl groups, an *n*-tetradecanoic acid moiety, or an *n*-tetradecanoic acid moiety substituted with one or two hydroxyl groups.

20

25-B1 Decapeptide Antifungal Agents

As used herein, "25-B1 decapeptide antifungal agent" refers to one or more members of a family of antifungal agents that has been isolated from the bacterium *Pseudomonas syringae*. A 25-B1 decapeptide antifungal agent is a *P. syringae*
25 lipodepsidecapeptide. Specifically, a 25-B1 decapeptide antifungal agent is a *P. syringae* lipodepsidecapeptide having a depsidecapeptide ring with the sequence:

Thr-Ala-Thr-Gln-Hse-Dhb-Ala-Dha-Thr-Arg (SEQ ID NO: 1)

Each of the 25-B1 decapeptide antifungal agents has the same cyclic peptide nucleus, but they differ in the hydrophobic side chain attached to this nucleus. The 25-B1 decapeptide
30 antifungal agents include 25-B1 decapeptide antifungal agent A.

The 25-B1 decapeptide antifungal agents include a fatty acid linked through an amide bond with the amino group of the N-terminal threonine. The fatty acid moiety of 25-B1 decapeptide antifungal agent A is an *n*-dodecanoic acid moiety.

5 Biological Activities of *P. syringae* Lipodepsidecapeptides

A *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, has several biological activities including killing and inhibiting activity of various fungi, such as fungal pathogens of plants and animals. In particular, a 25-B1 decapeptide antifungal agent is an active antimycotic agent against fungi that cause opportunistic
10 infections in immune compromised individuals. These fungi include *Cryptococcus neoformans*, *Histoplasma capsulatum* and various species of *Candida* including *C. parapsilosis* and *C. albicans*.

Pseudomonas syringae

15 *Pseudomonas syringae* include a wide range of bacteria that are generally associated with plants. Some of the *P. syringae* are plant pathogens, while others are only weakly pathogenic or are saprophytes. Many different isolates of *P. syringae* produce one or more cytotoxic agents that can help this bacterium survive in the wild where it must compete with fungi and other bacteria. The cytotoxic agents produced by *P. syringae*
20 include anti-fungal agents such as the *P. syringae* lipodepsidecapeptides, including 25-B1 decapeptide antifungal agent A, the pseudomycins, the syringomycins, the syringotoxins, and the syringostatins.

Isolated strains of *P. syringae* that produce one or more pseudomycins, syringomycins, syringotoxins, syringostatins are well-known to those skilled in the art.
25 Wild type strain MSU 174 and a mutant of this strain generated by transposon mutagenesis, MSU 16H (ATCC 67028) have been described in U.S. Patent No. 5,576,298, issued November 19, 1996 to G. Strobel et al.; Harrison et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," J. Gen. Microbiology 137, 2857-2865 (1991); and
30 Lamb et al., "Transposon mutagenesis and tagging of fluorescent pseudomonas:

Antimycotic production is necessary for control of Dutch elm disease," Proc. Natl. Acad. Sci. USA 84, 6447-6451 (1987). Methods for growth of various strains of *P. syringae* and their use in production of antifungal agents such as pseudomycins are also disclosed in U.S. Patent Application Serial No. PCT/US00/08728 by Matthew D. Hilton, et al.

5 entitled "Pseudomycin Production By *Pseudomonas Syringae*" submitted eventdate herewith and described below. The disclosures of the references cited in this paragraph are incorporated herein by reference.

Strains of *P. syringae* that are suitable for production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be isolated

10 from environmental sources including plants such as barley plants, citrus plants, and lilac plants, and from forest floor litter, soil, water, air, and dust. The present invention includes a strain, an isolate, and a biologically-purified culture of *P. syringae* that produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, in amounts greater than about 10 $\mu\text{g/mL}$, preferably from at least about 10

15 $\mu\text{g/mL}$ to about 50 $\mu\text{g/mL}$. Preferably, the biologically-purified culture of a microorganism is of *Pseudomonas syringae* strains MSU 16H, 25-B1, 67H1, 7H9-1, or a pseudomycin-producing mutant, variant, isolate, or recombinant of these strains. Cultures of MSU 16H are on deposit at Montana State University (Bozeman, Montana, USA) and available from the American Type Culture Collection (Parklawn Drive,

20 Rockville, MD, USA) Accession No. ATCC 67028.

A strain of *P. syringae* that is suitable for production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be isolated from environmental sources including plants, such as barley plants, citrus plants, and lilac plants, and also from sources such as soil, water, air, and dust. A preferred strain is

25 isolated from plants. These environmental isolates of *P. syringae* can be referred to as wild type. As used herein, "wild type" refers to a dominant genotype which naturally occurs in the normal population of *P. syringae* (i.e., strains or isolates of *P. syringae* that are found in nature and not produced by laboratory manipulation). As is the case with other organisms, the characteristics of the lipodepsidecapeptide-producing cultures

30 employed in this invention, *P. syringae* strains such as MSU 174, MSU 16H, MSU 206,

25-B1. and 7H9 are subject to variation. Thus, progeny of these strains, e.g., recombinants, mutants and variants, may be obtained by methods well-known to those skilled in the art.

Mutant strains of *P. syringae* are also suitable for production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A. As used herein, "mutant" refers to a sudden heritable change in the phenotype of a strain, which can be spontaneous or induced by known mutagenic agents, including radiation and various chemicals. Mutant *P. syringae* of the present invention can be produced using a variety of mutagenic agents including radiation such as ultraviolet light, and x-rays; chemical mutagens; site-specific mutagenesis; and transposon mediated mutagenesis. Examples of chemical mutagens are ethyl methyl sulfonate (EMS), diepoxyoctane, N-methyl-N-nitro-N'-nitrosoguanine (NTG), and nitrous acid.

P. syringae suitable for producing one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, according to the present invention can be generated by treating the bacteria with an amount of a mutagenic agent effective to produce mutants that overproduce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, or that produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, under advantageous growth conditions. While the type and amount of mutagenic agent to be used can vary, a preferred method is to serially dilute NTG to levels ranging from about 1 to about 100 $\mu\text{g/mL}$. Preferred mutants of the invention are those that overproduce 25-B1 decapeptide antifungal agent A, and grow in minimal medium. The mutants overproduce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, preferably from at least about 10 $\mu\text{g/mL}$ to about 50 $\mu\text{g/mL}$.

Environmental isolates, mutant strains, and other desirable strains of *P. syringae* can be subjected to selection for desirable traits of growth habit, growth medium, nutrient source, carbon source, growth conditions, and amino acid requirements. Preferably, a strain of *P. syringae* producing *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, is selected for growth on minimal defined medium. Preferred strains exhibit the characteristics of producing one or more *P. syringae*

lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, when grown on a medium including glycine plus, optionally, a lipid, a potato product, or both.

Recombinant strains can be developed by transforming the *P. syringae* strains, using procedures well-known to those skilled in the art. Through the use of recombinant technology, the *P. syringae* strains can be transformed to express a variety of gene products in addition to the antibiotics these strains produce. For instance, one can transform the strains with a recombinant vector that confers resistance to an antibiotic to which the strains are normally sensitive. Transformants thus obtained will produce not only a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, but also the resistance-conferring enzyme that allows selection of the transformed from wild type cells. Furthermore, using similar techniques, one can modify the present strains to introduce multiple copies of the endogenous lipodepsidecapeptide-biosynthesis genes to achieve greater lipodepsidecapeptide yield. Progeny, i.e. natural and induced variants, mutants and recombinants, of the *P. syringae* strains 25-B1, 67H1, and 7H9-1 which retain the characteristic of lipodepsidecapeptide production are part of this invention.

Growth of *Pseudomonas syringae*

As described herein, "aqueous nutrient media" refers to a water-base composition including minerals and organic compounds and their salts necessary for growth of the bacterium used in the present invention. Preferred nutrient media contain an effective amount of three or fewer amino acids, preferably, glutamic acid, glycine, histidine, or a combination thereof. In one embodiment, the medium contains an effective amount of glycine and, optionally, one or more of a potato product and a lipid. Glycine can be provided as a single amino acid or as part of a mixture of amino acids, such as hydrolyzed protein. Suitable lipids include soybean oil, fatty acids, or fatty acid esters. Suitable potato products include potato dextrose broth, potato dextrin, potato protein, or a commercial mashed potato mix food product. Preferred minerals in the nutrient medium include salt mixtures typically used in cell culture and fermentation, such as Czapek mineral salts, which includes KCl, $MgSO_4$, and $FeSO_4$. Organic compounds in the nutrient media preferably includes glucose and can optionally include soluble starch; other

like organic compounds can also be included. The pH of the medium is preferably between about 4 and 6.5, more preferably about 4.5 to about 5.7, most preferably about 5.2.

Although the amount of each ingredient in the nutrient broth is not typically
5 critical to growth of the bacteria or to production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, certain levels of nutrients are advantageous. A preferred amount of glycine is about 0.1 g/L to about 10 g/L, more preferably about 0.3 g/L to about 3 g/L, most preferably about 1 g/L. A preferred amount of lipid is about 1 g/L to about 10 g/L of an oil product such as soybean oil, more
10 preferably about 0.5 g/L to about 2 g/L of soybean oil. A preferred amount of a fatty acid or fatty acid ester is about 0.5 g/L to about 5 g/L. Preferred amounts of potato products include about 12 g/L to about 36 g/L, more preferably about 24 g/L of potato dextrose broth; about 5 g/L to about 50 g/L, preferably about 30 g/L of a commercial mashed potato mix; about 1 g/L to about 30 g/L, preferably about 20 g/L of potato dextrin; and/or
15 about 1 g/L to about 10 g/L, preferably about 4 g/L of potato protein. A preferred nutrient medium includes minerals, preferably, KCl at about 0.02 to about 2 g/L, more preferably about 0.2 g/L; MgSO₄, preferably MgSO₄•7H₂O, at about 0.02 to about 2 g/L, more preferably about 0.2 g/L; and FeSO₄, preferably FeSO₄•7H₂O, at about 0.4 to about 40 mg/L, more preferably about 4 mg/L. When present, soluble starch is preferably at about
20 0.5 to about 50 g/L, more preferably about 5 g/L. Glucose is preferably present at about 2 to about 80 g/L, more preferably about 20 g/L.

P. syringae are typically grown in the media described under conditions of controlled or regulated pH, and temperature. *P. syringae* grow and produce one or more cytotoxic agents at temperatures between about 15 °C and about 35 °C, preferably about
25 20 °C to about 30 °C, more preferably about 25 °C. *P. syringae* grow and produce one or more cytotoxic agents at pH between about 4 and about 9, more preferably between about 4 and about 6, most preferably from about 4.5 to about 5.5. Typically growth of *P. syringae* does not occur when the temperature is above about 37° C or below 10° C or when the pH is above about 9 or below about 4.

30

Method for Producing a *P. syringae* Lipodepsidecapeptide

To produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, from a wild type or mutant strain of *P. syringae*, the organism is cultured with agitation in an aqueous nutrient medium including an effective amount of three or fewer amino acids. The three or fewer amino acids are preferably glutamic acid, glycine, histidine, or a combination thereof. In one preferred embodiment, the amino acids include glycine and, optionally, one or more of a potato product and a lipid. Culturing is conducted under conditions effective for growth of *P. syringae* and production of a desired *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Effective conditions include a temperature of about 22° C to about 27° C, and a duration of about 36 hours to about 96 hours. When cultivated on media such as those described herein, *P. syringae* can grow at cell densities up to about 10-15 g/L dry weight and produce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, in a total amount at least about 10 µg/mL, preferably at least about 50 µg/mL.

Controlling the concentration of oxygen in the medium during culturing of *P. syringae* is advantageous for production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Preferably, oxygen levels are maintained at about 5% to about 50% saturation, more preferably about 30% saturation. Sparging with air, with pure oxygen, or with gas mixtures including oxygen can regulate the concentration of oxygen in the medium. Further, adjustment of the agitation rate can be used to adjust the oxygen transfer rate.

Controlling the pH of the medium during culturing of *P. syringae* is advantageous for production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. The pH of the culture medium can be maintained at less than about 6 and above about 4.

P. syringae can produce a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, when grown in batch culture. However, fed-batch or semi-continuous feed of glucose and, optionally, an acid or base, such as ammonium

hydroxide, to control pH, enhances production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. Production of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, by *P. syringae* can be further enhanced by using continuous culture methods in which glucose and, optionally, an acid or base, such as ammonium hydroxide, to control pH, are fed automatically. The pH is preferably maintained at a pH of about 5 to about 5.4, more preferably about 5.0 to about 5.2.

Choice of *P. syringae* strain can affect the amount and distribution of a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, produced by culturing under the conditions described herein. For example, strain 25 B1 can produce predominantly 25-B1 decapeptide antifungal agent A.

The cyclic decapeptide nucleus of the *P. syringae* lipodepsidecapeptides can be prepared by cleaving off the lipophilic moiety, such as by deacylation. Cleavage and deacylation methods are well-known to those skilled in the art, such as the use of deacylase enzymes.

Formulation and Antifungal Action of *P. syringae* Lipodepsidecapeptides

A *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, shows *in vitro* and *in vivo* activity and is useful in combating either systemic fungal infections or fungal skin infections. Accordingly, the present invention provides a method of inhibiting fungal activity including contacting a *P. syringae* lipodepsidecapeptide, such as a 25-B1 decapeptide antifungal agent, or a pharmaceutically acceptable salt thereof, with a fungus. A preferred method includes inhibiting growth or activity of various fungi such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, and species of *Candida* including *C. parapsilosis* and *C. albicans*. As used herein "contacting" a compound of the invention with a parasite or fungus refers to a union or junction, or apparent touching or mutual tangency of a compound of the invention with a parasite or fungus. However, the term contacting does not imply any mechanism of inhibition.

The present invention further provides a method of treating a fungal infection which includes administering an effective amount of a *P. syringae* lipodepsidecapeptide,

such as a 25-B1 decapeptide antifungal agent, or a pharmaceutically acceptable salt thereof, to a host in need of such treatment. A preferred method includes treating an infection by various fungi such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, and strains of *Candida* including *C. parapsilosis* and *C. albicans*. When administered in an effective antifungal amount, a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, reduces the burden of a fungal infection, reduces symptoms associated with the fungal infection, and can result in elimination of the fungal infection.

Some patients in need of antifungal therapy with a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, have severe symptoms of infection, such as high fever, and are likely to be in intensive or critical care. Various fungi can cause such serious infections. *Candida spp.*, for example, causes mucosal and serious systemic infections and may exist as azole- or polyene-resistant strains. *Aspergillus* causes life-threatening systemic infections. *Cryptococcus* is responsible for meningitis. Such serious fungal infections may occur in immune compromised patients, such as those receiving organ or bone marrow transplants, undergoing chemotherapy for cancer, recovering from major surgery, or suffering from HIV infection. For such patients, antifungal therapy typically includes intravenous administration, of a formulation of one or more *P. syringae* lipodepsidecapeptides (e.g., the 25-B1 decapeptide antifungal agents) over several days to halt or retard the infection.

With respect to antifungal activity, the term "effective amount" means an amount of a compound of the present invention which is capable of inhibiting fungal growth or activity, or reducing symptoms of the fungal infection. For most fungal infections reduction of symptoms of the infection includes reduction of fever, return to consciousness, and increased well being of the patient. Preferably, symptoms are reduced by killing the fungus to eliminate the infection or to bring the infection to a level tolerated by the patient or controlled by the patient's immune system. As used herein "inhibiting" refers to inhibiting fungal activity, including stopping, retarding or prophylactically hindering or preventing the growth or any attending characteristics and results from the existence of a fungus.

Typically, the compositions will be administered to a patient (human or other animal, including mammals such as cats, horses and cattle and avian species) in need thereof, in an effective amount to inhibit the fungal infection. The dose administered will vary depending on such factors as the nature and severity of the infection, the age and
5 general health of the host and the tolerance of the host to the antifungal agent. The particular dose regimen likewise may vary according to such factors and may be given in a single daily dose or in multiple doses during the day. The regimen may last from about 2-3 days to about 2-3 weeks or longer. A typical daily dose (administered in single or divided doses) will contain a dosage level of from about 0.01 mg/kg to about 100 mg/kg
10 of body weight of an active compound of this invention. Preferred daily doses generally will be from about 0.1 mg/kg to about 60 mg/kg and ideally from about 2.5 mg/kg to about 40 mg/kg. For serious infections, the compound can be administered by intravenous infusion using, for example, 0.01 to 10 mg/kg/hr of the active ingredient.

The present invention also provides pharmaceutical formulations useful for
15 administering the antifungal compounds of the invention. Accordingly, the present invention also provides a pharmaceutical formulation including one or more pharmaceutically acceptable carriers, diluents, vehicles, excipients, or other additives and one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A. The active ingredient in such formulations includes from 0.1% to 99.9% by
20 weight of the formulation, more generally from about 10% to about 30% by weight. By "pharmaceutically acceptable" it is meant that the carrier, diluent or excipient is compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The formulation can include additives such as various oils, including those of
25 petroleum, animal, vegetable or synthetic origin, for example, peanut oil, soybean oil, mineral oil, and sesame oil. Suitable pharmaceutical excipients include starch, cellulose, glucose, lactose, sucrose, gelatin, malt, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, and ethanol. The compositions can be subjected to conventional pharmaceutical expedients,
30 such as sterilization, and can contain conventional pharmaceutical additives, such as

preservatives, stabilizing agents, wetting, or emulsifying agents, salts for adjusting osmotic pressure, and buffers. Suitable pharmaceutical carriers and their formulations are described in Martin, "Remington's Pharmaceutical Sciences," 15th Ed.; Mack Publishing Co., Easton (1975); see, e.g., pp. 1405-1412 and pp. 1461-1487.

5 The term "pharmaceutically acceptable salt", as used herein, refers to salts of the compounds of the above formula that are substantially non-toxic to living organisms. Typical pharmaceutically acceptable salts include those salts prepared by reaction of the compounds of the present invention with a mineral or organic acid or an inorganic base. Such salts are known as acid addition and base addition salts.

10 Acids commonly employed to form acid addition salts are mineral acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, and phosphoric acid, and organic acids such as p-toluenesulfonic, methanesulfonic acid, oxalic acid, p-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, and acetic acid. Examples of such pharmaceutically acceptable salts are the sulfate,
15 pyrosulfate, bisulfate, sulfite, bisulfite, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, propionate, decanoate, caprylate, acrylate, formate, isobutyrate, caproate, heptanoate, propiolate, oxalate, malonate, succinate, suberate, sebacate, fumarate, maleate, butyne-1,4-dioate, hexyne-1,6-dioate, benzoate, chlorobenzoate, methylbenzoate,
20 dinitrobenzoate, hydroxybenzoate, methoxybenzoate, phthalate, sulfonate, xylenesulfonate, phenylacetate, phenylpropionate, phenylbutyrate, citrate, lactate, gamma-hydroxybutyrate, glycollate, tartrate, methanesulfonate, propanesulfonate, naphthalene-1-sulfonate, naphthalene-2-sulfonate, and mandelate. Preferred pharmaceutically acceptable acid addition salts are those formed with mineral acids such as hydrochloric
25 acid and hydrobromic acid, and those formed with organic acids such as maleic acid and methanesulfonic acid.

 Base addition salts include those derived from inorganic bases, such as ammonium or alkali or alkaline earth metal hydroxides, carbonates, and bicarbonates. Such bases useful in preparing the salts of this invention thus include sodium hydroxide, potassium
30 hydroxide, ammonium hydroxide, potassium carbonate, sodium carbonate, sodium

bicarbonate, potassium bicarbonate, calcium hydroxide, and calcium carbonate. The potassium and sodium salt forms are particularly preferred.

It should be recognized that the particular counterion forming a part of any salt of this invention is not of a critical nature, so long as the salt as a whole is pharmacologically acceptable and as long as the counterion does not contribute undesired qualities to the salt as a whole.

A *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, may be administered parenterally, for example using intramuscular, subcutaneous, or intra-peritoneal injection, nasal, or oral means. In addition to these methods of administration, a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, may be applied topically for superficial skin infections or to inhibit fungal growth in the mucus.

For parenteral administration the formulation includes one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, and a physiologically acceptable diluent such as deionized water, physiological saline, 5% dextrose and other commonly used diluents. The formulation may contain a cyclodextrin and/or a solubilizing agent such as a polyethylene glycol or polypropylene glycol or other known solubilizing agent. Such formulations may be made up in sterile vials containing the antifungal and excipient in a dry powder or lyophilized powder form. Prior to use, a physiologically acceptable diluent is added and the solution withdrawn via syringe for administration to the patient.

The present pharmaceutical formulations are prepared by known procedures using known and readily available ingredients. In making the compositions of the present invention, the active ingredient is generally admixed with a carrier, or diluted by a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet, paper or other container. When the carrier serves as a diluent, it may be a solid, semi-solid or liquid material which acts as a vehicle, excipient or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols, (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active

compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, or sterile packaged powders.

For oral administration, the antifungal compound is filled into gelatin capsules or formed into tablets. Such tablets may also contain a binding agent, a dispersant or other
5 suitable excipients suitable for preparing a proper size tablet for the dosage and a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A. For pediatric or geriatric use the antifungal compound may be formulated into a flavored liquid suspension, solution or emulsion. A preferred oral formulation is linoleic acid, cremophor RH-60 and water and preferably in the amount (by volume) of 8% linoleic
10 acid, 5% cremophor RH-60, 87% sterile water and a *P. syringae* lipodepsidecapeptide, such as 25-B1 decapeptide antifungal agent A, in an amount of from about 2.5 to about 40 mg/ml.

For topical use the antifungal compound may be formulated with a dry powder for application to the skin surface or it may be formulated in a liquid formulation including a
15 solubilizing aqueous liquid or non-aqueous liquid, e.g., an alcohol or glycol.

Uses of Formulations of a *P. syringae* Lipodepsidecapeptide

The present invention also encompasses a kit including the present pharmaceutical compositions and to be used with the methods of the present invention. The kit can contain
20 a vial which contains a formulation of the present invention and suitable carriers, either dried or in liquid form. The kit further includes instructions in the form of a label on the vial and/or in the form of an insert included in a box in which the vial is packaged, for the use and administration of the compounds. The instructions can also be printed on the box in which the vial is packaged. The instructions contain information such as sufficient dosage
25 and administration information so as to allow a worker in the field to administer the drug. It is anticipated that a worker in the field encompasses any doctor, nurse, or technician who might administer the drug.

The present invention also relates to a pharmaceutical composition including a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide
30 antifungal agent A, and that is suitable for administration by injection. According to the

invention, a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be used for manufacturing a composition or medicament suitable for administration by injection. The invention also relates to methods for manufacturing compositions including a formulation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, in a form that is suitable for oral or topical administration. For example, a liquid or solid formulation can be manufactured in several ways, using conventional techniques. A liquid formulation can be manufactured by dissolving the one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, in a suitable solvent, such as water, at an appropriate pH, including buffers or other excipients.

Agricultural Uses

Antibiotics produced from *P. syringae* NRRL B-12050 have been demonstrated to effectively treat Dutch elm disease. (see, e.g., U.S. Patent Nos. 4,342,746 and 4,277,462) In particular, *P. syringae* MSU 16H has been shown to confer a greater protection than the wild-type strain in elms infected with *Ceratocystis ulmi*, the causal agent of Dutch elm disease. (see e.g., Lam et al, *Proc. Natl. Sci. USA*, 84, 6447-6451 (1987)). More extensive tests on field-grown elms confirmed the phenomenon of biocontrol at the prophylactic level. Hence, the lipodepsidecapeptides of the present invention may be useful as a preventative treatment for Dutch Elm disease. The pseudomycins have been shown to be toxic to a broad range of plant-pathogenic fungi including *Rhynchosporium secalis*, *Ceratocystis ulmi*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Verticillium albo-atrum*, *Verticillium dahliae*, *Thielaviopsis basicola*, *Fusarium oxysporum* and *Fusarium culmorum*. (see Harrison, L., et al., "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing broad-spectrum antifungal activity," *J. General Microbiology*, 7, 2857-2865 (1991).) Consequently, one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A (including hydrates, solvates, and esters thereof) may be useful in the treatment of fungi in plants (in particular, *V. albo-atrum*, *Rhizoctonia solani* and *F. oxysporum*) either as a direct treatment or preventative treatment. Generally, the infected plants are treated by injecting or spraying an aqueous suspension of the lipodepsidecapeptide compounds into

or onto the plant. Means of injection are well-known to those skilled in the art (e.g., gouge pistol). Any means of spraying the suspension may be used that distributes an effective amount of the active material onto the plant surface. The suspension may also include other additives generally used by those skilled in the art, such as solubilizers, stabilizers, wetting agents, and combinations thereof.

Treatment of the plant may also be accomplished using a dry composition containing one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A. The dry formulation may be applied to the plant surface by any means well-known to those skilled in the art, such as spraying or shaking from a container.

The present invention may be better understood with reference to the following examples. These examples are intended to be representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

EXAMPLES

Biological Materials on Deposit

P. syringae MSU 16H is publicly available from the American Type Culture Collection, Parklawn Drive, Rockville, MD, USA as Accession No. ATCC 67028. *P. syringae* strains 25-B1, 7H9-1, and 67 H1 were deposited with the American Type Culture Collection on March 23, 2000 and were assigned the following Accession Nos.:

| | |
|-------|------------------------|
| 25-B1 | Accession No. PTA-1622 |
| 7H9-1 | Accession No. PTA-1623 |
| 67 H1 | Accession No. PTA-1621 |

Example 1 – Production of 25-B1 Antifungal Agent A

Fermentation methods were developed for producing a lipodepsidecapeptide antifungal agent, 25-B1 decapeptide antifungal agent A, in the fermentation broth of a *Pseudomonas syringae* strain.

Materials and Methods

Preparation of inoculum: An aliquot of *P. syringae* strain 25-B1 cells stored in the vapor phase of liquid nitrogen was thawed and used to inoculate two 900 mL portions of CSM broth. CSM broth was composed of (g/L): dextrose (5), maltose (4), Difco Tryptic Soy Broth (30), Difco yeast extract (3), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2). Approximately 0.5 mL of cells was used to inoculate each 900 mL portion of medium contained in a two liter flask. Flasks were incubated with shaking for 24 hours at 25°C. The contents of two flasks were combined to inoculate a 150 liter fermentor containing 115 liters of sterile fermentation broth.

Fermentation Stage: Fermentation broth was composed of (g/L): dextrose (20), soluble starch (5), Basic American Foods Country Style Potato Pearls instant mashed potatoes (30), glycine (1), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), KCl (0.2), and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.004) in tap water. The pH was adjusted to 5.2 before sterilization. Fermentation was carried out at 25°C for 68 hr. Dissolved oxygen was maintained at or above 30% of air saturation by continuous adjustment of air flow and impeller agitation rate. The pH was maintained between 4.0 and 5.4 through the addition of either H_2SO_4 or NaOH.

Several variations of the simple batch process were also found to produce the novel cyclic peptide product. Dextrose may be fed to the fermentors starting 24 hours after initial inoculation at a rate of 60 mL per hour. Feeding may be continued throughout the course of the fermentation. Alternatively, a process has been used where dissolved oxygen is maintained at 5% of air saturation starting 24 hours after inoculation and continuing until the end of the fermentation period. Maintenance of dissolved oxygen at 5% was achieved through addition of inert nitrogen gas (N_2) to the air supply leading to the fermentor. In all cases, gas was supplied through a single submerged sparger tube with an opening positioned just below the bottom agitator turbine in the fermentor.

Results and Conclusion

Several fermentation methods produce 25-B1 decapeptide antifungal agent A from *P. syringae*

Example 2 – Isolation and Purification of 25-B1 Antifungal Agent A

Methods were developed for isolation and purification of a lipodepsidecapeptide antifungal agent. 25-B1 decapeptide antifungal agent A. from the fermentation broth of a *Pseudomonas syringae* strain.

5

Materials and Methods

The whole fermentation broth produced according to Example 1, typically 100 L after harvest, was filtered through a Membralox™ ceramic filter (0.45 μ m). The resulting solid slurry was extracted with an equal volume of acetone containing 0.1% TFA for 90 min. The acetone extract was separated by filtration and evaporated *in vacuo* to an aqueous solution.

This solution was combined with the filtrate obtained from the ceramic filtration of the whole broth and charged on to an Amberchrom™ CG 300sd resin column (4 L) packed in water. The column was initially washed with 0.2% acetic acid (pH 4-8) until the effluent showed pH 4.5 followed by 10 L of 22% acetonitrile containing 0.2% acetic acid (pH 4.8). Then the column was eluted with a linear gradient of 22-35% acetonitrile containing 0.2% acetic acid (32 L) and 35% acetonitrile containing 0.2% acetic acid (8 L) with 400 ml/min flow rate. Fractions 11-16 were combined (4.8 L), concentrated *in vacuo* to 100 ml and centrifuged.

The supernatant was separated and chromatographed over an Amberchrom CG 300sd column (1 L) using a linear gradient of 25-35% acetonitrile containing 0.2% acetic acid (pH 4.8) with 50 ml/min flow rate. Fractions 20-25 (1.2 L) were combined and rechromatographed over a reversed-phase column (NovaPak C₁₈, 6 μ m, 40X300 mm, flow rate 40 ml/min, linear gradient 30-60% acetonitrile containing 0.2% TFA) to yield 21 mg of a compound (89% purity by UV).

The ESIMS data showed a possible [M+H]⁺ peak at m/z 1165.7, which is different from the known antifungal agents that have been found thus far from *P. Syringae*. The ¹H NMR spectrum showed signals reminiscent of pseudomycin-like lipopeptide but indicated the presence of more than one compound. In order to obtain 25-B1 decapeptide antifungal agent A in high purity for structure determination and antifungal activity, an

additional broth from 4X100 L fermentation was processed as described above and in addition, the final purification was carried out on a reversed-phase column [Rainin C₁₈, 6 μ m, 24X250 mm, 0.1% TFA-acetonitrile-MeOH (8:1:1 to 4:3:3) gradient elution for 60 min: (4:3:3 to 10:45:45) gradient elution for 30 min] to afford 42 mg of 25-B1
5 decapeptide antifungal agent A (93% purity by UV).

Results and Conclusion

HPLC methods similar to those used to purify other lipodepsipeptide antifungal agents resulted in purification of 25-B1 decapeptide antifungal agent A from fermentation
10 broth.

Example 3 – Determination of the Structure of 25-B1 Antifungal Agent A

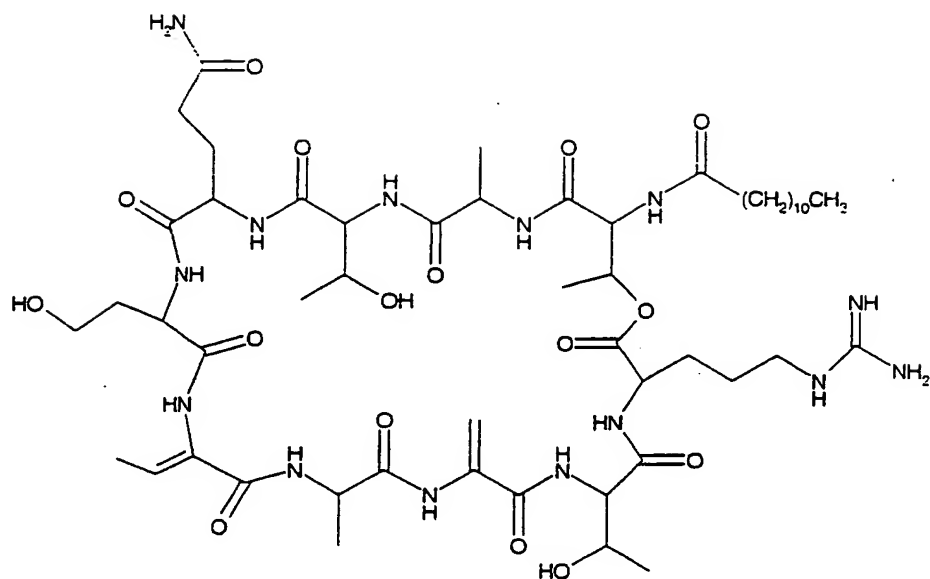
Mass spectrometry and NMR determined the structure of a lipodepsidecapeptide antifungal agent, 25-B1 decapeptide antifungal agent A.
15

Methods and Results

The molecular formula of 25-B1 decapeptide antifungal agent A was determined by high resolution FABMS as C₅₂H₈₈N₁₄O₁₆[m/z 1165.6581 for C₅₂H₈₉N₁₄O₁₆(M+H)⁺, Δ +0.9 ppm].

20 In accordance with this formula the ¹³C and DEPT NMR spectra showed 50 distinct resonances, which included twelve carbonyl carbons, five olefinic carbons, four oxygenated sp³ carbons, eight typical amino acid α -carbons, fifteen methylene carbons and six methyl carbons. Among these, one of the methyl carbon signals at δ 16.7 and one of the methylene carbon signals at δ 28.9 each constituted a set of degenerate carbons.
25 thus accounting for the total number of 52 carbons observed in the molecular formula.

Detailed analysis of ¹H, ¹³C, and 2D NMR (DQCOSY, TOCSY, HMQC, HMBC and ROESY) data enabled to determine the structure of 25-B1 decapeptide antifungal agent A (IA) and unambiguously assign all the protons and carbons (Table 1).



IA

Table 1. ^1H and ^{13}C NMR Chemical Shifts of 25-B1 decapeptide antifungal agent A in DMSO- d_6

| Amino acid | Position | δ_{H} | δ_{C} |
|------------|------------|---------------------|---------------------|
| Arg | NH | 8.13 | - |
| | CO | - | 170.4 |
| | α | 4.23 | 51.7 |
| | $\beta 1$ | 1.69 | 28.2 |
| | $\beta 2$ | 1.63 | |
| | γ | 1.46 | 24.7 |
| | δ | 3.08 | 40.2 |
| | NH | 7.59 | - |
| Thr-l | ϵ | - | 156.7 |
| | NH | 7.83 | - |
| | CO | - | 169.8 |
| | α | 4.23 | 59.5 |
| | β | 4.00 | 66.4 |
| | γ | 1.08 | 19.7 |
| Dha | | | |
| | NH | 9.01 | - |
| | CO | - | 163.7 |
| | α | - | 135.3 |
| | $\beta 1$ | 5.86 | 106.4 |
| | $\beta 2$ | 5.61 | |

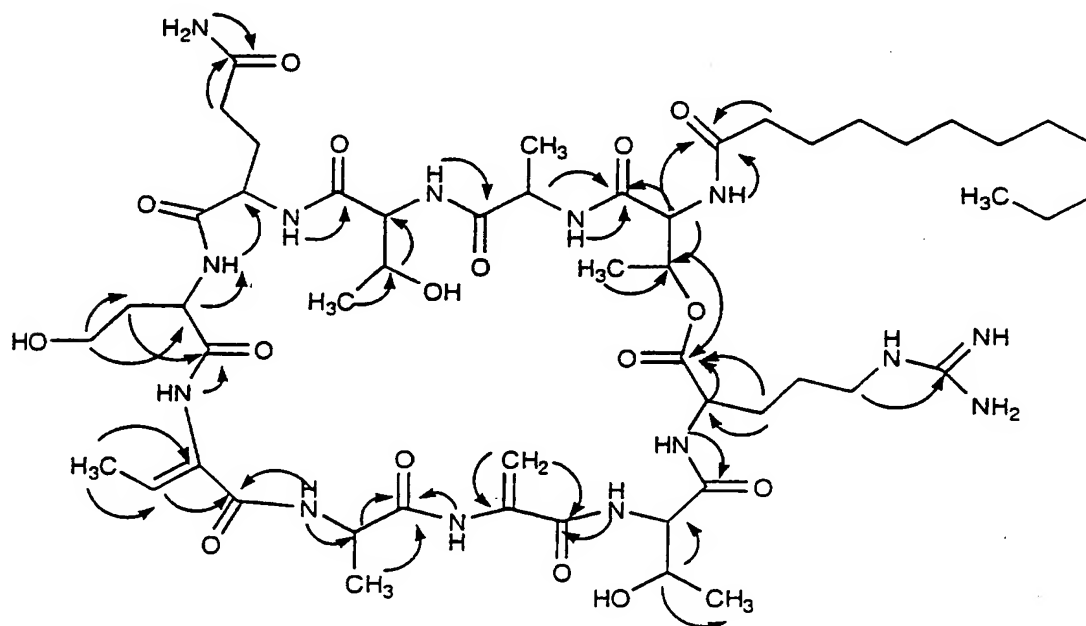
| Amino acid | Position | δ_{H} | δ_{C} |
|------------|-----------------|---------------------|---------------------|
| Ala-1 | NH | 7.96 | - |
| | CO | - | 171.6 |
| | α | 4.22 | 49.9 |
| | β | 1.31 | 16.7 |
| Dhb | NH | 9.07 | - |
| | CO | - | 164.0 |
| | α | - | 130.0 |
| | β | 6.40 | 128.5 |
| | γ | 1.61 | 12.9 |
| HSer | NH | 8.22 | - |
| | CO | - | 171.1 |
| | α | 4.22 | 51.4 |
| | β_1 | 1.82 | 34.0 |
| | β_2 | 1.76 | |
| | γ | 3.47 | 57.3 |
| Gln | NH | 7.59 | - |
| | CO | - | 171.9 |
| | α | 4.31 | 52.0 |
| | β_1 | 1.90 | 27.9 |
| | β_2 | 1.73 | |
| | γ | 2.11 | 31.1 |
| | CO | - | 173.9 |
| | NH ₂ | 7.13 | - |
| | | 6.72 | |
| Thr-2 | NH | 7.80 | - |
| | CO | - | 169.9 |
| | α | 4.11 | 58.4 |
| | β | 4.11 | 66.0 |
| | γ | 1.01 | 19.8 |
| Ala-2 | NH | 8.18 | - |
| | CO | - | 172.4 |
| | α | 4.32 | 48.5 |
| | β | 1.16 | 17.6 |

| Amino acid | Position | δ_H | δ_C |
|------------|----------|------------|--------------------------------------|
| Thr-3 | NH | 7.93 | - |
| | CO | - | 168.6 |
| | α | 4.47 | 55.6 |
| | β | 4.94 | 70.5 |
| | γ | 1.12 | 16.7 |
| Side chain | | | |
| | CO | | 172.2 |
| | 2 | 2.08 | 35.1 |
| | 3 | 1.44 | 25.1 |
| | 4 | 1.21 | 28.5 |
| | 5-10 | 1.21 | 31.1, 28.9X2, 28.8, 28.7, 28.6 |
| | 11 | 1.21 | 22.0 |
| | 12 | 0.83 | 13.9 |

The results from 1H , DQCOSY and TOCSY spectra measured in DMSO- d_6 at 35°C revealed the presence of spin systems for seven commonly occurring amino acid residues – two alanines, one arginine, one glutamine and three threonines, and three less commonly occurring amino acids – one dehydroalanine (Dha), one dehydroaminobutyric acid (Dhb) and one homoserine. The less commonly occurring amino acid residues, viz. Dha, Dhb and homoserine, were identified by the cross peaks observed in the TOCSY spectrum from the amide protons resonating at δ 9.01 (brs), 9.07 (brs) and 8.22 to protons resonating at δ 5.86, 5.61 (δ_C 106.4), 6.40 (δ_C 128.5), 1.61 (δ_C 12.9) and 4.22 (δ_C 51.4), 1.82, 1.76 (δ_C 34.0), 3.47 (δ_C 57.3), respectively. Consistent with this the ^{13}C NMR spectrum displayed eight proton bound α -carbon signals for the saturated amino acid residues and two quaternary α -carbons for the unsaturated amino acids Dha and Dhb. Of the twelve amide or ester type carbonyls, nine were assigned to the eight saturated amino acid residues (two to glutamine), two (δ 164.0 and 163.7 ppm) to Dha and Dhb. The remaining one carbonyl group was assigned to the dodecanoyl side chain, the presence of which is discerned from the terminal methyl signal (δ_H 0.83 and δ_C 13.9) and 10 methylene signals in the ^{13}C NMR spectrum (Table 1).

The molecular formula requires sixteen degrees of unsaturation. The ten amino acids and the dodecanoyl side chain accounted for fifteen sites of unsaturation indicating that 25-B1 decapeptide antifungal agent A is a monocyclic decapeptide. Comparison of the chemical shift of the β -protons of threonines (δ_H 4.94, 4.11 and 4.00) indicated that the proton resonating at δ 4.94 was attached to a carbinol which is modified to an ester or a lactone. That this was so and 25-B1 decapeptide antifungal agent A is a depsipeptide was evidenced by the HMBC and ROESY data. These data also established the amino acid sequence and the location of the dodecanoyl side chain in 25-B1 decapeptide antifungal agent A.

With amino acid Arg as a starting point, the long range $^1H - ^{13}C$ correlations observed in the HMBC spectrum between the amide proton and the adjacent amino acid carbonyl and or α -carbon (see Scheme I below) unambiguously established the amino acid sequence Arg-NH/Thr-CO, Thr-NH/Dha-CO, Dha-NH/Ala-CO, Ala-NH/Dhb-CO, Dhb-NH/Hse-CO, Hse-NH/Glu- α -C, Glu-NH/Thr-CO, Thr-NH/Ala CO and Ala-NH/Thr-CO.

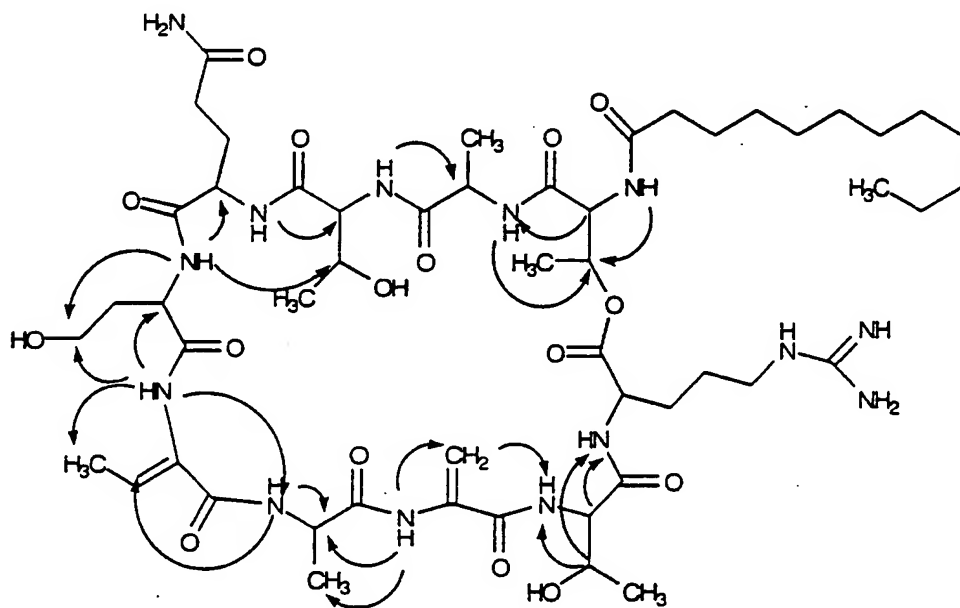


HMBC (H \rightarrow C) Correlations

Scheme I

The NH of Thr adjacent to Ala did not show a long range $^1\text{H} - ^{13}\text{C}$ correlation to the carbonyl of Arg residue, instead it showed a correlation to a carbonyl assigned to the dodecanoyl side chain. The absence of Thr-NH/Arg-CO correlation and presence of a correlation between the Thr- β -H (δ_{H} 4.94, δ_{C} 70.5)/Arg-CO clearly established an ester linkage between the Thr- β -OH and Arg-COOH. Consistent with these assignments are the ROESY correlations that were observed between the amide protons and the adjacent amino acid α -protons (see Scheme II below).

10



Selected ROESY Correlations

Scheme II

Conclusions

Compound 25-B1 decapeptide antifungal agent A represents a novel class of lipodepsipeptide which possesses several amino acid residues that are not present in any of the pseudomycins, syringomycins, syringotoxin and syringostatins produced by different isolates of *P. syringae*. The new depsipeptide is composed of ten amino acids

which is also a departure from the pseudomycins and syringomycins which possess only nine amino acid residues. Unlike the pseudomycin natural products, the new depsipeptide does not include chlorothreonine which is suspected to be the cause for irritation at the injection site of pharmaceutical formulations containing pseudomycin compounds.

5

Example 4 -- Antifungal activity of 25-B1 Decapeptide Antifungal Agent A

The antifungal studies were conducted using a microtiter broth dilution assay according to National Committee for Clinical Laboratory Standards guidelines in 96 well microtiter plates. Sabourauds and dextrose broth was adjusted to contain 2.5×10^4 conida/ml. Test compound was dissolved in water and tested in two-fold dilutions starting with the highest concentration of 20 $\mu\text{g/ml}$. Plates were incubated at 35°C for 48 hr. The results in Table 2 show the minimal inhibitory concentration (MIC) of the compound that completely inhibited growth compared to untreated growth controls.

15

Table 2. Antifungal activity of 1

| Organism | MIC ($\mu\text{g/ml}$) |
|--------------------------------|--------------------------|
| <i>Candida albicans</i> | 10 |
| <i>C. parapsilosis</i> | 10 |
| <i>Cryptococcus neoformans</i> | 1.25 |
| <i>Aspergillus fumigatus</i> | >20 |
| <i>Histoplasma capsulatum</i> | 20 |

The presence or amount of one or more *P. syringae* lipodepsipeptides, such as 25-B1 decapeptide antifungal agent A, can be determined by measuring the antifungal activity of a preparation. Antifungal activity can be determined in vitro by obtaining the minimum inhibitory concentration (MIC) of the preparation using a standard agar dilution test or a disc diffusion test. A preparation of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, can be an extract of a cell culture, or a more purified mixture. A typical fungus employed in testing antifungal activity is *C. albicans*. Antifungal activity is considered significant when the test preparation causes 10-12 mm diameter zones of inhibition on *Candida albicans* x657 seeded agar plates.

Example 5 -- Isolation, Characterization and Mutagenesis of *Pseudomonas syringae*

Environmental isolates and mutants of *P. syringae* were produced and employed
5 in production of antifungal agents.

Materials and Methods

Strains MSU 174 and MSU 16-H were isolated and characterized as described in
U.S. Patent No. 5,576,298, issued November 19, 1996 to G. Strobel et al.; Harrison et al.,
10 "Pseudomycins, a family of novel peptides from *Pseudomonas syringae* possessing
broad-spectrum antifungal activity," J. Gen. Microbiology **137**, 2857-2865 (1991); and
Lamb et al., "Transposon mutagenesis and tagging of fluorescent pseudomonas:
Antimycotic production is necessary for control of Dutch elm disease," Proc. Natl. Acad.
Sci. USA **84**, 6447-6451 (1987). The disclosures of the references cited in this paragraph
15 are incorporated herein by reference.

Additional strains were derived from such wild type and transposon generated
mutants by chemical mutagenesis. Strains subjected to mutagenesis include MSU 174,
MSU 16H, and 25-B1. The strain to be mutagenized was grown in a medium containing
potato product, then divided into the medium including 0, 1, 2, 4, 16, or 32 μ M of the
20 chemical mutagen 1-methyl-3-nitro-1-nitrosoguanidine (NTG or MNNG). These cells
were then frozen for future screening and selection.

Mutagenized cells were selected for desirable growth conditions and/or production
of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal
agent A. Chemically mutagenized cells of *P. syringae*, such as mutagenized strain 25-B1,
25 were thawed and diluted to 6 cells/mL in N21SM medium (Table 3). This medium
sometimes contained one or more components for selection, such as varying
concentrations of phosphate. A 50 μ L volume of mutagenized cells was dispensed into a
well of a 96-well round bottom microtiter plate for a delivery of an average of 0.3
cells/well. Typically, silicone oil was added to each well to minimize evaporation. The
30 plates were incubated with shaking for 6 to 12 days at 25 °C.

Table 3 -- The Composition of N21SM Medium

| INGREDIENT | GRAMS PER LITER |
|----------------------------------|--------------------|
| Glucose | 20 |
| Ammonium Sulfate | 0.5 |
| Monosodium Glutamate | 2 |
| L-Histidine | 2 |
| Glycine | 0.5 |
| Soluble Starch | 5 |
| KH ₂ PO ₄ | 0.2 |
| Czapek Mineral Salts Solution | 2 mL |
| MES Buffer | 9.8 |
| Adjust pH to 5.0 | |

After this incubation, an aliquot, typically 5 μ L, from each well was serially diluted (e.g. 1:56, 1:196, 1:320, 1:686, and/or 1:1715) and evaluated for activity against *Candida albicans* in a liquid microtiter plate bioassay. The plates were incubated at 37 °C overnight and the wells were scored for inhibition of *C. albicans* growth. Suitable strains were picked, inoculated into CSM medium (Table 4), and grown for 1 to 3 days at 25 °C.

Table 4. Complete Streptomyces Medium (CSM)

| Component | Concentration (g/L) |
|--------------------------------------|---------------------|
| Glucose | 5 |
| Maltose | 4 |
| Difco Tryptic Soy Broth | 30 |
| Difco Yeast Extract | |
| MgSO ₄ ·7H ₂ O | 2 |
| No pH adjustment | |

The selected strains were preserved and inoculated into fermentation bottles containing 13mL of N21SM medium and grown for approximately 66 hours at 25 °C. An aliquots was removed from this fermentation, extracted for 1 hour with a volume of acetonitrile equal to the volume of the aliquot, centrifuged, and decanted for HPLC analysis of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, as described in Examples 1-3. Strains producing one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, were reisolated, re fermented, and prepared for growth on a larger scale.

Results

Strains exhibiting production of one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A, were produced using the methods described above.

Conclusion

The selection methods and criteria disclosed herein are effective for producing strains of *P. syringae* that grow on minimal medium and produce one or more *P. syringae* lipodepsidecapeptides, such as 25-B1 decapeptide antifungal agent A.

Example 6 – Formulations Including *P. syringae* Lipodepsidecapeptide

The following formulation examples are illustrative only and are not intended to limit the scope of the invention in any way. The term "active ingredient" means a *P. syringae* lipodepsidecapeptide or a pharmaceutically acceptable salt thereof.

5

Formulation 1

Hard gelatin capsules are prepared using the following ingredients:

| Ingredient | Quantity (mg/capsule) |
|--------------------|--------------------------|
| Active ingredient | 250 |
| Starch, dried | 200 |
| Magnesium stearate | 10 |
| Total | 460 mg |

10 **Formulation 2**

A tablet is prepared using the ingredients below. The components are blended and compressed to form tablets each weighing 665 mg.

| Ingredient | Quantity (mg/capsule) |
|-----------------------------|--------------------------|
| Active ingredient | 250 |
| Cellulose, microcrystalline | 400 |
| Silicon dioxide, fumed | 10 |
| Stearic acid | 5 |
| Total | 665 mg |

15 **Formulation 3**

An aerosol solution is prepared containing the following components. The active compound is mixed with ethanol and the mixture added to a portion of the propellant 22, cooled to -30°C . and transferred to a filling device. The required amount is then fed to a stainless steel container and diluted with the remainder of the propellant. The valve units
20 are then fitted to the container.

| Component | Weight (g) |
|--|------------|
| Active ingredient | 0.25 |
| Methanol | 27.75 |
| Propellant 22 (Chlorodifluoromethane) | 74.00 |
| Total | 100.00 |

Formulation 4

Tablets, each containing 60 mg of active ingredient, are made as follows:

5

| | |
|--|--------|
| Active ingredient | 60 mg |
| Microcrystalline cellulose | 45 mg |
| Polyvinylpyrrolidone (as 10% solution in water) | 4 mg |
| Sodium carboxymethyl starch | 4.5 mg |
| Magnesium stearate | 0.5 mg |
| Talc | 1 mg |
| Total | 150 mg |

The active ingredient, starch and cellulose are passed through a No. 45 mesh U.S. sieve and mixed thoroughly. The aqueous solution containing polyvinyl-pyrrolidone is mixed with the resultant powder, and the mixture then is passed through a No. 14 mesh U.S. sieve. The granules so produced are dried at 50o C. and passed through a No. 18 mesh U.S. sieve. The sodium carboxymethyl starch, magnesium stearate and talc, previously passed through a No. 60 mesh U.S. sieve, are then added to the granules which, after mixing, are compressed on a tablet machine to yield tablets each weighing 150 mg.

15

Formulation 5

Capsules, each containing 80 mg of active ingredient, are made as follows:

| | |
|----------------------------|--------|
| Active ingredient | 80 mg |
| Starch | 59 mg |
| Microcrystalline cellulose | 59 mg |
| Magnesium stearate | 2 mg |
| Total | 200 mg |

The active ingredient, cellulose, starch and magnesium stearate are blended, passed through a No. 45 mesh U.S. sieve, and filled into hard gelatin capsules in 200 mg quantities.

5

Formulation 6

Suppositories, each containing 225 mg of active ingredient, are made as follows:

| | |
|---------------------------------|----------|
| Active ingredient | 225 mg |
| Saturated fatty acid glycerides | 2,000 mg |
| Total | 2,225 mg |

10

The active ingredient is passed through a No. 60 mesh U.S. sieve and suspended in the saturated fatty acid glycerides previously melted using the minimum heat necessary. The mixture is then poured into a suppository mold of nominal 2 g capacity and allowed to cool.

15 Formulation 7

Suspensions, each containing 50 mg of active ingredient per 5 ml dose, are made as follows:

| | |
|--------------------------------|---------|
| Active ingredient | 50 mg |
| Sodium carboxymethyl cellulose | 50 mg |
| Syrup | 1.25 ml |
| Benzoic acid solution | 0.10 ml |
| Flavor | q.v. |
| Color | q.v. |
| Purified water to total | 5 ml |

The active ingredient is passed through a No. 45 mesh U.S. sieve and mixed with the sodium carboxymethyl cellulose and syrup to form a smooth paste. The benzoic acid solution, flavor and color are diluted with a portion of the water and added, with stirring. Sufficient water is then added to produce the required volume.

5

Formulation 8

An intravenous formulation may be prepared as follows. The solution of these ingredients generally is administered intravenously to a subject at a rate of 1 ml per minute.

| | |
|-------------------|----------|
| Active ingredient | 100 mg |
| Isotonic saline | 1,000 mg |

10

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

WE CLAIM:

1. An isolated *P. syringae* depsidecapeptide, or a pharmaceutically acceptable salt, ester, or hydrate thereof, comprising a depsidecapeptide ring wherein the depsidecapeptide ring comprises arginine, threonine, homoserine, dehydroaminobutyric acid, and dehydroalanine, and a lactone is formed from a carboxyl group of the arginine and a hydroxyl group of the threonine.

2. The *P. syringae* depsidecapeptide of claim 1, wherein the depsidecapeptide ring has a sequence:
Thr -Ala-Thr-Gln-Hse-Dhb-Ala-Dha-Thr-Arg (SEQ ID NO: 1).

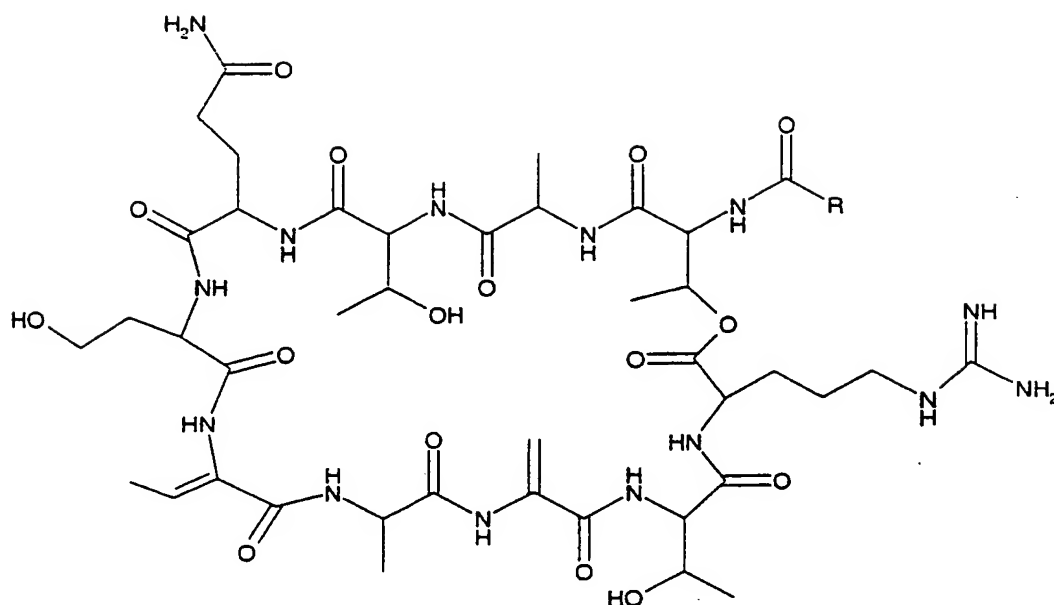
3. The *P. syringae* depsidecapeptide of claim 1, wherein the *P. syringae* depsidecapeptide is a *P. syringae* lipodepsidecapeptide.

4. The *P. syringae* depsidecapeptide of claim 3, wherein the *P. syringae* lipodepsidecapeptide comprises a fatty acid moiety coupled to an amino group of the threonine by an amide bond.

5. The *P. syringae* depsidecapeptide of claim 4, wherein the fatty acid moiety is a decanoic acid moiety, a decanoic acid moiety substituted with one or two hydroxyl groups, a dodecanoic acid moiety, a dodecanoic acid moiety substituted with one or two hydroxyl groups, a tetradecanoic acid moiety, or a tetradecanoic acid moiety substituted with one or two hydroxyl groups.

6. The *P. syringae* depsidecapeptide of claim 5, wherein the fatty acid moiety is an *n*-dodecanoic acid moiety.

7. The *P. syringae* depsidecapeptide of claim 3, wherein the *P. syringae* lipodepsidecapeptide is represented by the formula:



where R is a lipophilic moiety, or a pharmaceutically acceptable salt, ester, or hydrate thereof.

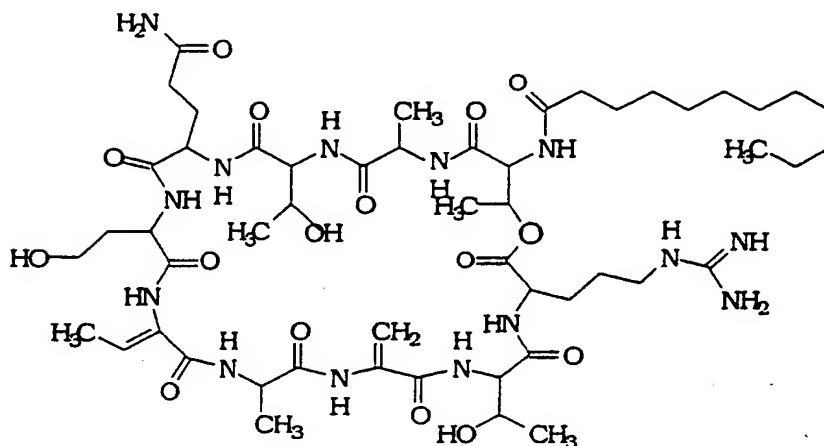
5

8. The *P. syringae* depsidecapeptide of claim 7, wherein the lipophilic moiety is selected from the group consisting of C₉-C₁₅ alkyl, C₉-C₁₅ hydroxyalkyl, C₉-C₁₅ dihydroxyalkyl, C₉-C₁₅ alkenyl, C₉-C₁₅ hydroxyalkenyl, and C₉-C₁₅ dihydroxyalkenyl.

10

9. The *P. syringae* depsidecapeptide of claims 8, wherein the lipophilic moiety is C₉-C₁₅ alkyl.

10. An isolated *P. syringae* depsidecapeptide having the formula:



- 5 or a pharmaceutically acceptable salt, ester, or hydrate thereof.

11. A method of inhibiting fungal activity comprising contacting a fungus with an isolated *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

- 10 12. The method of claim 11, wherein the fungus comprises *Candida parapsilosis*, *Candida albicans*, *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

13. A method of reducing the symptoms of a fungal infection in a patient in need thereof comprising:

- 15 administering to the patient an effective amount of a composition comprising an isolated *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

14. The method of claim 13, wherein reducing the symptoms comprises decreasing a burden of a fungal infection.

15. The method of claim 13, wherein the fungal infection comprises infection by *Candida parapsilosis*, *Candida albicans*, *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

5 16. The method of claim 13, further comprising the steps of:
determining the need for administering isolated *P. syringae* depositeptide; and
monitoring the patient for relief of symptoms of the fungal infection.

17. The method of claim 13, wherein administering comprises parenteral
10 administration about 1 to about 3 times per day of about 0.1 to about 5 mg/kg of isolated
P. syringae depositeptide.

18. The method of claim 13, wherein reducing the symptoms of a fungal
infection comprises reducing fever and increasing general well being of the patient.

15 19. Use of an isolated *P. syringae* depositeptide of Claims 1, 2, 3, 4, 5, 6,
7, 8, 9 or 10 in the manufacture of a medicament for the treatment of a fungal infection.

20. The use of Claim 19 wherein the fungal infection comprises infection by
20 *Candida parapsilosis*, *Candida albicans*, *Cryptococcus neoformans*, or *Histoplasma capsulatum*.

21. A method for producing one or more *P. syringae* depositeptides
comprising the steps of:
25 culturing a biologically pure culture of *Pseudomonas syringae* in nutrient medium
comprising three or fewer amino acids at a pH of about 4 to about 6.5 until one or more *P.*
syringae depositeptide is produced at a concentration of at least about 10 µg/mL; and
recovering one or more *P. syringae* depositeptides, or a pharmaceutically
acceptable salt, ester, or hydrate thereof, from the culture.

30

22. The method of claim 21, wherein the amino acid comprises glutamic acid, glycine, histidine, or a combination thereof.

23. The method of claim 22, wherein the amino acid comprises glycine.

24. The method of claim 22, wherein the nutrient medium further comprises soluble starch, yeast extract, or a combination thereof and a lipid.

25. The method of claim 24, wherein the nutrient medium comprises a potato product and a lipid selected from the group consisting of soybean oil, fatty acids, and fatty acid esters.

26. The method of claim 25, wherein the potato product comprises potato dextrose broth, mashed potato mix, potato dextrin, potato protein, or a combination thereof.

27. The method of claim 21, wherein during the culturing step the dissolved oxygen concentration is maintained at about 5% to 30%.

28. The method of claim 21, wherein the culturing step further comprises feeding of glucose, ammonium hydroxide, or a combination thereof.

29. The method of claim 21, wherein the *P. syringae* depsidecapeptide produced is a *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10.

30. The method of claim 21, wherein the *Pseudomonas syringae* comprises a strain derived from strain MSU 16H, MSU 174, or MSU 206.

31. The method of claim 21, wherein the *Pseudomonas syringae* comprises strain MSU 16H, strain 25-B1, strain 67H1, or strain 7H9-1.

32. The method of claim 31, wherein the *Pseudomonas syringae* comprises strain 25-B1.

5 33. A *P. syringae* depsidecapeptide of Claim 7 prepared by the methods of Claims 21, 22, 23, 24, 25, 26, 27, 28, 30, 31 or 32.

34. A method for treating or preventing fungal growth in a plant comprising contacting a fungus with a *P. syringae* depsidecapeptide of Claims 1, 2, 3, 4, 5, 6, 7, 8, 9
10 or 10.

35. The method of Claim 33 wherein the fungus is *Rynchosporium secalis*, *Ceratocystis ulmi*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Verticillium albo-atrum*, *Verticillium dahliae*, *Thielaviopsis basicola*, *Fusarium oxysporum* or *Fusarium culmorum*.

15

36. The method of Claim 34 wherein the fungus is *V. albo-atrum*, *Rhizoctonia solani* or *F. oxysporum*.

SEQUENCE LISTING

<110> Eli Lilly and Company

<120> Antifungal Agents Isolated from Pseudomonas Syringae

<130> X-11015 Sequence Listing

<140>

<141>

<150> 60/129,446

<151> 1999-04-15

<160> 1

<170> PatentIn Ver. 2.1

<210> 1

<211> 10

<212> PRT

<213> Pseudomonas syringae

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<223> Xaa at position 6 is dehydroaminobutyric acid.

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<223> Xaa at position 8 is dehydroalanine.

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Thr Ala Thr Gln Xaa Xaa Ala Xaa Thr Arg

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X-11015

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| 0-1 | Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis) | |
| 0-1-1 | Prepared using | PCT-EASY Version 2.90 (updated 08.03.2000) |
| 0-2 | International Application No. | |
| 0-3 | Applicant's or agent's file reference | X-11015 |
| 1 | The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on: | |
| 1-1 | page | 19 |
| 1-2 | line | 20 |
| 1-3 | Identification of Deposit | |
| 1-3-1 | Name of depositary institution | American Type Culture Collection |
| 1-3-2 | Address of depositary institution | 10801 University Blvd., Manassas, Virginia 20110-2209 United States of America |
| 1-3-3 | Date of deposit | 23 March 1999 (23.03.1999) |
| 1-3-4 | Accession Number | ATCC PTA-1622 |
| 1-4 | Additional Indications | NONE |
| 1-5 | Designated States for Which Indications are Made | all designated States |
| 1-6 | Separate Furnishing of Indications These indications will be submitted to the International Bureau later | NONE |
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| 2-1 | page | 19 |
| 2-2 | line | 21 |
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| 2-3-1 | Name of depositary institution | American Type Culture Collection |
| 2-3-2 | Address of depositary institution | 10801 University Blvd., Manassas, Virginia 20110-2209 United States of America |
| 2-3-3 | Date of deposit | 23 March 2000 (23.03.2000) |
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| 3-1 | page | 19 |
| 3-2 | line | 22 |
| 3-3 | Identification of Deposit | |
| 3-3-1 | Name of depositary institution | American Type Culture Collection |
| 3-3-2 | Address of depositary institution | 10801 University Blvd., Manassas, Virginia 20110-2209 United States of America |
| 3-3-3 | Date of deposit | 23 March 2000 (23.03.2000) |
| 3-3-4 | Accession Number | ATCC PTA-1621 |
| 3-4 | Additional Indications | NONE |
| 3-5 | Designated States for Which Indications are Made | all designated States |
| 3-6 | Separate Furnishing of Indications | NONE |
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/08724

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K11/02 A61K38/15 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| A | US 4 416 874 A (KAPLAN ET AL.) 22 November 1983 (1983-11-22) the whole document | 1-36 |
| A | WO 95 11310 A (CHEIL FOODS AND CHEMICALS INC.) 27 April 1995 (1995-04-27) the whole document | 1-36 |



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

23 August 2000

Date of mailing of the international search report

30/08/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Masturzo, P

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/08724

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
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| WO 9511310 A | 27-04-1995 | KR 9709888 B | 19-06-1997 |
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